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Inhibition of Pannexin 1 reduces tumorigenic properties of human melanoma

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Abstract

Pannexin 1 (PANX1) is a channel-forming glycoprotein that allows the passage of important signaling molecules, including ATP. We examined PANX1 levels in a panel of human melanomas and evaluated its potential as an effective target for melanoma therapy. We are the first to report endogenous PANX1 levels in multiple human melanoma cell lines, patient-derived melanoma biopsies, and primary melanoma cells. Treatment with two PANX1 channel blockers, Carbenoxolone (CBX) and Probenecid (PBN), on A375 and A375-MA2 melanoma cells significantly reduced cell growth and migration, and increased the production of melanin, a marker for a melanocytic-like phenotype. Daily treatment with CBX or PBN onto A375-MA2 cells grown on the chorioallantoic membrane of a chicken embryo model significantly reduced primary melanoma tumour growth. Our findings suggest a possible dysregulation of PANX1 in human melanomas that may contribute to malignant behaviour and provides support for PANX1 channel blockers as a potential treatment for melanoma.

Keywords

Pannexins, PANX1, melanoma, Carbenoxolone, Probenecid, Chick-CAM assay, tumour growth, cancer treatment

Co-Authorship Statement

All cell culture, immunoblot, immunofluorescence, and chick-CAM experiments were performed by Taylor J. Freeman. Danielle Johnston assisted with the isolation of primary melanoma cells from fresh patient biopsies and assisted in all chick-CAM experiments. Daniel Nouri Nejad taught methods for the growth curves, scratch assays, and melanin extractions, and helped with cracking eggs for chick-CAM experiments. Rafael Sanchez-Pupo assisted with egg cracking in all chick-CAM experiments and data analysis. Dr. Silvia Penuela designed the thesis, supervised the experimental procedures, data analysis and assisted with the editing process of the final manuscript.

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List of Abbreviations

α-MSH	α- melanocyte stimulating hormone
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BRAF	V-Raf murine sarcoma viral oncogene homolog B1; serine/threonine-protein kinase
CBX	Carbenoxolone
CAM	Chorioallantoic membrane
COPII	Coat protein II
CSD	Chronically sun damaged
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
Cx46	Connexin 46
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gly0	Un-glycosylated pannexin
Gly1	High mannose species of pannexin
Gly2	Complex glycosylation species of pannexin
KO	Knockout
L10	L10BIOBR-GFP
LHSC	London Health Sciences Centre
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor

MFQ	Mefloquine
MITF	Microphthalmia-associated transcription factor
MUP	Melanomas of unknown primary
NF1	Neurofibromin 1
NRAS	Isoform of *RAS* oncogene; encodes G proteins with GTPase activity
OICR	Ontario Institute for Cancer Research
PANX	Human pannexin
Panx	Mouse or generic pannexin
PBN	Probenecid
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
REK	Rat epidermal keratinocyte
Trova	Trovafloxacin
UV	Ultra-violet

Chapter 1

1 Introduction

1.1 Pannexins

Pannexins (Panx in rodents, or PANX in human), are a novel family of channel-forming glycoproteins composed of three family members: Panx1, Panx2, Panx3 [1-3]. Discovered in the year 2000, pannexins were identified in vertebrates based on their limited sequence homology to the invertebrate gap junction protein, innexins, and were named based on their nearly ubiquitous distribution throughout the animal kingdom [1]. Sequence analyses of pannexins have a predicted topography of four transmembrane domains, two extracellular loops, a single intracellular loop, and carboxy- and amino-terminals ends that extend into the cytoplasm [4]. Panx2 (~73kDa) contains 677 amino acid residues and is the largest protein member of the pannexin family, whereas Panx1 (~48kDa) and Panx3 (~45kDa) are smaller with only 426 and 392 amino acids, respectively [2, 4-6]. Panx1 and Panx3 are both located on chromosome 11 and share the most sequence homology (41%) out of the three family members [4].

Pannexins are integral membrane proteins that are co-translationally inserted into the endoplasmic reticulum (ER) [7, 8]. Glycosylation is an important post-translational modification that regulates cellular localization of each pannexin [7, 9]. Research performed by Penuela et al. (2007) used sequence analysis and site-directed mutagenesis to predict and confirm the site for glycosylation in the second extracellular loop of Panx1 (N254) and the first extracellular loop of Panx3 (N71) [7]. The site for Panx2 glycosylation is predicted to be on the first extracellular loop (N86) and has yet to be confirmed [9]. Pannexins may exist as un-glycosylated proteins (Gly0) or as high mannose species (Gly1) that predominantly localize to intracellular compartments including the ER [9-11]. Panx1 and Panx3 follow the ER-Golgi secretory pathway where assembly and vesicular transport of newly synthesized pannexin proteins occurs through a COPII (coat protein II)-dependent mechanism [8]. Through this pathway, Panx1 and Panx3 are transported to the Golgi apparatus and acquire more complex glycosylation

extensions (Gly2). Pannexins are preferentially localized to the cell membrane in this form [9]. Early on in this pathway, pannexin monomers oligomerize and form non-selective, hexameric channels that allow for the passage of molecules up to 1kDa in size including ions and ATP [3, 12, 13]. Previous work has identified protein interactions among the pannexins regulated by the extent of glycosylation, which can potentially constitute heteromeric channels of Panx1 and Panx2 or Panx1 and Panx3; however, no apparent interaction has been found between Panx2 and Panx3 [2, 7, 9]. In addition, caspase 3 and 7 have been shown to cleave the inhibitory carboxy-terminal end of Panx1 and Panx2 [14, 15]. *In vitro* analyses did not find Panx3 to be a target for caspase-mediated digestion [15]. Caspase cleavage has been found to open and constitutively activate Panx1 channels [16]. For example, reports in apoptotic cells have shown that ATP release from caspase-cleaved PANX1 channels can act as a 'find-me' signal for the recruitment of immune cells for the purposes of cell clearance [14].

Although no sequence homology exists between pannexins and connexins, pannexins show structural similarities to connexin hemichannels and were initially proposed as a new gap junction family [4, 5]. However, several differences between these proteins have been reported in the literature. Studies have revealed an even distribution of pannexins throughout the cell membrane, whereas connexins show selective clustering where cells come into contact [9, 17]. Additionally, pannexins contain carbohydrate modifications that likely act to repel adjacent channels and membranes thereby preventing the close contact that would be necessary for pannexins to form gap junctions [18, 19]. Furthermore, the regulation of pannexin and connexin channels likely differs. Panx1 and Panx3 can remain at the cell surface for time periods greater than 21 hours, in contrast with the extremely short half-life of connexins (1-5 hours) [9, 10, 20].

Pannexins have been identified in different cell types and tissues [2]. In comparison to the widespread expression of Panx1 which will be discussed in the following section, Panx2 and Panx3 expression is more restricted. Panx3 has been identified in the skin, bone and cartilage and has been found to play an important role in keratinocyte, chondrocyte and osteoblast differentiation via ATP release [21-23]. Panx3 has additionally been located in the lung, liver, kidney, spleen and thymus of 3-week-old

mice [7]. Panx2 was originally thought to exist primarily in the central nervous system; however, Le Vasseur et al. (2014) has recently identified Panx2 expression in many different mouse tissues including the skin, skeletal muscle, and the spleen [4, 24]. Loss of Panx2 has been found to accelerate N2a cell progression into a neuronal phenotype, identified by morphology changes and increased levels of the mature neuronal marker NeuN [25]. In addition to Panx1, Panx2 was found to contribute to ischemic brain damage in a stroke model [26]. This report found that both Panx1 and Panx2 had to be ablated from mice for a neuroprotective effect to occur, due to a compensation effect by both pannexin species [26]. Other studies have also identified potential Panx3 compensation for the loss of Panx1, as higher levels of this protein have been identified in a Panx1 knockout mouse [27].

Coordinated cellular communication is essential for the proper development of different tissues. Since pannexin channels have an important role in paracrine and autocrine signaling, they present as novel targets in many diseases where cellular communication may be altered [6, 28].

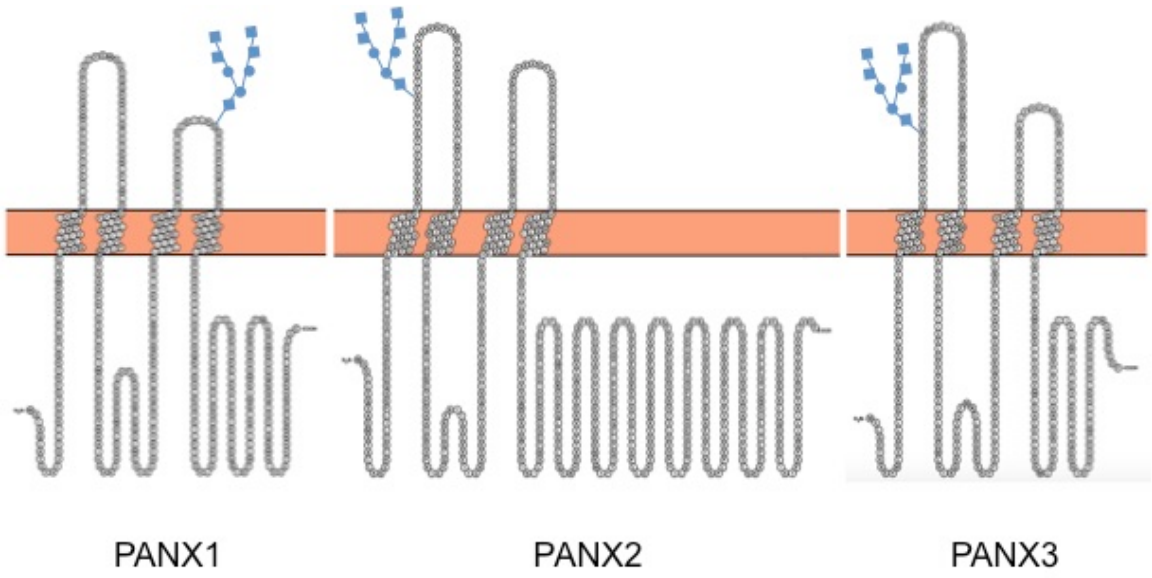

Figure 1.1. Proposed tetra-spanning topology of PANX1, PANX2 and PANX3. Each protein is thought to have an intracellular amino (NH_2) and carboxyl (COOH) termini, four transmembrane domains, one intracellular loop and two extracellular loops. The glycosylation sites (blue) on PANX1 and PANX3 are located at N254 and N71. The site for Panx2 glycosylation is predicted to be on the first extracellular loop (N86) and requires confirmation. Images were developed using Protter [29].

1.2 Pannexin 1

Pannexin 1 (Panx1 in rodents, or PANX1 in human) monomer proteins oligomerize into hexameric channels intracellularly (i.e. in the ER [30]) and can be trafficked to the cell surface where they have been primarily characterized as conduits for ATP release [3, 14, 31]. Under normal physiological conditions, Panx1 channels are thought to be closed and tightly regulated. These channels can be activated via a number of different stimuli including mechanical stimulation [31], high extracellular potassium [32], intracellular calcium [33], hypoxemia [34], and voltage stimulation [35, 36]. In addition, Panx1 channels can be closed by negative feedback from extracellular ATP [37] and by PANX1 channel blockers [36, 38]. Research suggests that regulation of the channel may occur through the carboxy-terminal end, which acts in a ball-and-chain manner to reversibly

close the pore [16]. However, cleavage of the carboxy-terminal end by caspase 3 or 7 can lead to constitutive opening and activation of the channel [14, 16]. Other reports provide further insight into channel regulation by describing residues in the first extracellular loop of Panx1 that may have a role in the channel's gating mechanism upon channel blockade [36].

Panx1 is the most characterized species of the three pannexins, likely due to its widespread expression in mammalian tissue [2, 6, 39]. For example, Northern blot analysis has identified PANX1 expression in the heart, liver, testis, small intestine, ovary, placenta, thymus, lung, pancreas, spleen, colon and blood [4]. Panx1 expression has also been identified in different structure of the mouse brain including the cerebral cortex, hippocampus, cerebellum, and brain stem [40]. More recent study has additionally confirmed the presence of the Panx1 protein in several tissues within a 3-week-old C57/BL6 mice, including the skin, kidney, tail and ear cartilage, and the ventricles [7]. Due to its widespread expression, Panx1 has been described in both normal and disease states throughout the body. One of its more extensively studied roles involves ATP-release for the purpose of purinergic signaling. Panx1-mediated ATP release has been shown to initiate and propagate Ca^{2+} waves and can modulate the release of nitric oxide (NO) onto smooth muscle surrounding blood vessels to cause vasodilation when erythrocytes respond to shear stress or low oxygen [33, 41]. Release of ATP from these large pore channels has also been shown to participate in cell-to-cell signaling in taste buds and regulation of defense mechanisms of the airway, including mucocillary clearance [42, 43]. Additionally, pro-inflammatory cytokines and ATP can be released from Panx1 channels to facilitate a viral infection following an interaction between HIV-1 and CD4+ cells [44, 45]. In the brain, research has also shown that Panx1 channels are activated from increased levels of extracellular K+ released during intense neuronal activity, leading to the release of ATP from the channel that contributes to neuronal hyperactivity in seizures of juvenile mice [46]. Finally, Panx1 has a noted role in skin development and the malignancy of several cancers including melanoma [27, 39, 47]; however, this will be discussed in more detail in the following sections.

1.3 Skin Biology

The skin is the largest organ of the body. It forms a barrier against the external environment that protects from invasion of pathogens and microbes, temperature extremes, and attenuates physical and chemical assaults on the body [48, 49]. The skin also contains a unique system to combat UV radiation and limits the loss of water and electrolytes from bodies [48, 49]. Skin is composed of three separate layers: the hypodermis, the dermis, and the epidermis. The hypodermis contains mainly fatty tissue and functions to connect the dermis to the underlying skeletal muscle. The dermis is derived from the mesoderm during development and is a thick layer of connective tissue that includes the neural, lymphatic, vascular and secretory components of the skin [49, 50]. Fibroblasts are the main cell type of this layer and give the cell its elasticity and tensile strength by secreting and modulating components of the extracellular matrix including collagen [49]. In addition, multiple structures can be found in the dermal tissue including secretory glands, sensory nerve receptors for touch and pressure, and hair follicles, which provide a protective niche for keratinocyte and melanocyte stem cells [49, 51].

The epidermis is the smallest layer of skin located on top of the dermal tissue. The epidermis develops from embryo ectoderm and goes through programmed differentiation to form four or five distinct layers [50]. Approximately 95% of the epidermis is made up of keratinocytes while the remaining 5% is composed of melanocytes [49]. The bottom, single cell layer of the epidermis is known as the *stratum basale*. Keratinocytes contain stem like properties and are the predominant cell type of this layer [52]. Merkel cells and melanocytes may also be found in this layer and are derived from neural-crest cells of the ectoderm during development [50]. The *stratum spinosum* is the largest layer of the epidermis and consists of polyhedral keratinocytes and few antigen-presenting Langerhans' cells [53]. Finally, the upper layers of the epidermis consist of the *stratum granulosum,* the *stratum lucidum* (only in thick skin) and the *stratum corneum*. In the top layers, highly differentiated keratinocytes do not divide, have a flattened morphology, lack cytoplasmic organelles, produce keratin and are referred to as corneocytes [52]. These cells provide a protective barrier against physical, bacterial, chemical and

environmental agents. The skin is continuously replenishing new cells. It takes approximately 28 days for a human keratinocyte cell to differentiate and travel from the *stratum basale* to the *stratum corneum* [54].

Although the melanocytes represent a small percentage of cells that make up the skin, they have an essential role at protecting our skin against harmful UV-related damage. Melanocytes originate from the cells of the ectodermal germ layer of an embryo known as neural crest cells [55]. These cells are present on the edges of the neural plate, have a mesenchymal phenotype and will form melanocytes if they originate from the cranial area or trunk of the developing embryo and follow a primarily dorsolateral migratory pathway during development [55]. Melanoblasts are melanocyte precursor cells that do not produce melanin [55]. As melanoblasts travel to their set destination, they express melanogenic genes that are regulated by MITF (microphthalmia-associated transcription factor), a master melanocytic-lineage transcription factor [56]. MITF expression is activated as melanoblasts develop from neural crest cells and is essential for survival as the cells migrate [57]. MITF is also expressed in mature states and can influence melanocyte proliferation and survival, dendrite formation, and melanin production through downstream factors [55, 56]. Mature melanocytes will reside in multiple locations of the body including the skin, the eyes, hair follicles, oral mucosa, meninges, the anogenital tract, and the inner ear [55, 58].

Melanocytes located in the epidermis are situated in the *stratum basale* layer with cellular projections incorporated into the *stratum spinosum* layer [49]. Projections from each melanocyte contact keratinocyte cells at a ratio of 1:10-40 cells, respectively [55]. Melanocytes divide infrequently and function to produce a pigment molecule known as melanin [59]. The production of melanin occurs when UV-exposure causes the release of α-melanocyte stimulating hormone (α-MSH) from keratinocytes and binds to the melanocortin 1 receptor (MC1R) located in melanocytes that is responsible for regulating melanin synthesis through downstream factors [60-62]. For example, MITF is located downstream of MC1R and can regulate enzymes involved in pigmentation [56]. Melanosomes synthesize and store complex pigments referred to as melanin that can be brown-black (eumelanin) or red-yellow (pheomelanin) depending on the presence of

thiols during synthesis [55, 63]. Mature melanosomes are translocated along microtubules and actin in the cell and are released from melanocytic dendrites into the extracellular space where they are taken up by keratinocytes [63-65]. Melanin will then disperse around the perinuclear region of the keratinocytes in a UV-exposure dependent manner in order to block and protect keratinocyte DNA from UV-damage [55, 64, 66].

Our skin has developed an eloquent system for proper development and protection from harmful UV-damage. However, there is a need to better understand proteins involved in these processes as any disruption to these molecular pathways could result in malignancies.

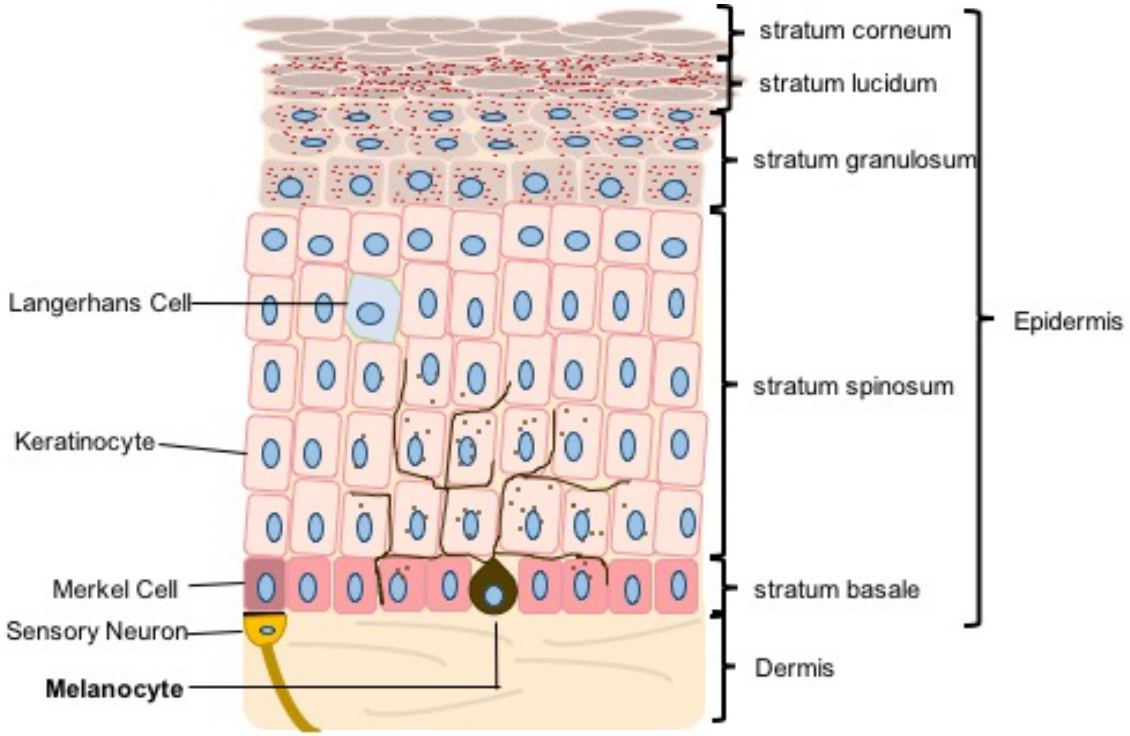

Figure 1.2. The epidermis is the top layer of skin and is composed of five layers including the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. The epidermis contains keratinocytes, melanocytes, Merkel cells, and Langerhans cells.

1.4 Pannexins in Skin

A coordinated set of events involving different signaling pathways is required for proper development of the skin. Panx1 and Panx3 are reported to be expressed in skin and have different roles in skin development [21, 47]. As previously mentioned, endogenous Panx2 levels have recently been identified in murine skin and require further characterization [24]. Panx1 has been detected throughout the epidermis and dermis, in cells including keratinocytes, L10 melanocytes (L10BIOBR-GFP), fibroblasts and cells of the inner and outer sheath of hair follicle roots [21, 27, 67]. Its expression has been primarily described in the *stratum granulosum* and *spinosum* and is absent in the cornified layers of the epidermis [7, 21]. Expression analyses performed in dorsal mouse skin further characterized Panx1 localization to the cell surface of basal keratinocytes [27]. Panx1 becomes more intracellularly localized as keratinocytes differentiate and move to the surface of the epidermis. Western blot and immunofluorescence data have shown high Panx1 levels in murine skin starting at E13.5 that decreases during development and skin maturation. This gradual reduction in expression begins after the mouse reaches 4-weeks of age and is significantly reduced in mice that are 12-weeks-old [27, 47]. Collectively, this suggests that Panx1 may be more important during early skin development but its role may be less essential in adult skin.

Panx3 has also been detected in both human facial and murine dorsal skin [21, 47]. Interestingly, Panx3 displays a dispersed distribution throughout the epidermal layer and has been detected in keratinocytes, the inner and outer root sheaths of hair follicles, sebaceous and eccrine glands [21, 47]. In one report, Panx3 expression in the basal layer of the epidermis did not co-localize to a melanocytic marker in human skin; however, further study is required to assess Panx3 expression in melanocytes [21]. In comparison to Panx1, Panx3 exhibits a predominant intracellular localization that remains high as the skin matures [21, 47].

Due to differences in expression and localization between Panx1 and Panx3 channels, it is not surprising that the role of each protein differs slightly from one another in essential keratinocyte processes including skin differentiation, maintenance or regeneration [7, 9].

Ectopic expression of Panx1 and Panx1-GFP in rat epidermal keratinocytes (REKs) resulted in reduced cell proliferation and dysregulated keratinocyte differentiation, described by a disorganized architecture of organotypic epidermis, a reduction in layer thickness and increased levels of cytokeratin 14, which is a common marker of basal, undifferentiated keratinocytes [47]. Although overexpression of Panx3 in REK cells also reduced cell proliferation, the differentiation process of REK cells and the organotypic epidermis was relatively unaffected as evaluated via architecture of the epidermis and through markers of differentiation [47]. These results suggest that Panx1 may be important during the development of healthy epidermal tissue, since Panx1 levels need to be downregulated in mature skin to increase differentiation in developing keratinocytes. Panx3 expression was relatively stable throughout skin development and therefore may be more important in the maintenance of healthy epidermal tissue.

Research performed on a global *Panx1* knockout (KO) mouse showed a viable, fertile mouse with no overt phenotype when compared to controls, which is surprising considering the extensive expression and function of PANX1 throughout the body [27]. Upon further investigation of this model, reduced epidermal and dermal tissue layers were discovered, highlighting the importance of Panx1 in skin architecture [27, 68]. When dermal punch biopsies were applied to the dorsal skin of *Panx1* KO mice, there was a significant delay in wound closure and increased levels of wound fibrosis when compared to control mice. In addition, primary fibroblasts show increased levels of proliferation and a reduced ability to differentiate into contractile myofibroblasts when Panx1 is depleted [27]. Although low Panx1 levels are found in mature skin, it is apparent that Panx1 has an essential role in wound healing when external challenges are applied.

1.5 Pannexin 1 and Cancer

Given its widespread expression and different roles throughout systems of the body, Panx1 has also been linked both directly and indirectly to multiple human diseases [6, 39]. The majority of cancer reports indicate that increased levels, expression or an enhanced function of the pannexin channel correlates with the onset or progression of the

disease [39]. Three different leukemic cell lines were found to have a 20-fold increase in PANX1 levels compared to normal human blood-derived T lymphoblast cells [69]. In gliomas, Panx1 expression in rat C6 cells accelerated P2X7-mediated assembly of multicellular tumour aggregates [70]. A separate report found a significant reduction in U87-MG human glioma cell proliferation following *PANX1* siRNA transfection [71]. Similarly, when Panx1 levels were reduced using shRNA knockdown in BL6 mouse melanoma cells, the cells revert to a more melanocytic-like phenotype and show decreased levels of cell growth and migration [67]. These trends in Panx1 dysregulation have also been described at the transcriptional level. Over-expression of Panx1 has been indicated across highly aggressive multiple myeloma cell lines and in mouse hepatocarcinoma cell lines [72, 73]. Hepatocarcinoma cell lines with high Panx1 expression were found to have an increased ability to metastasize. In line with these studies, the Human Protein Atlas has reported PANX1 levels in the majority of cancers types, with a few notable exceptions including testis and prostate cancer. Although most reports discuss the role of Panx1 in cancer progression, other research has shown that chemotherapeutic agents can exploit Panx1 channels to increase the anti-tumour response [69]. Here, pro-apoptotic chemotherapy drugs, including topoisomerase inhibitors, activated Panx1 channels in acute lymphocytic leukemia cells through caspase-3-mediated cleavage [69]. Activation of Panx1 channels released ATP, ADP, and AMP into the extracellular environment thereby mediating an immune response that targets dying cells [69].

Mutations in PANX1 have also been correlated with malignant phenotypes in cancers. A recent study found a mutated form of PANX1 (*C268T*) in highly metastatic breast cancer cells. The resulting truncated form of the protein ($PANX1^{1-89}$) was unable to function on its own. However, when co-expressed with a full-length form of the protein, the channel exhibited a gain-of function effect leading to augmented ATP release that acts on P2Y-purinergic receptors [74]. Cells become deformed as they migrate into the vasculature triggering the mechanical activation of PANX1 channels. Activation of the PANX1-purinergic-mediated pathway suppressed apoptosis and reduced cell death during cell migration, thereby enhancing the metastatic survival of breast cancer cells [74]. With these results, this study uncovered a role for PANX1 in cancer metastasis. This study also

revealed the clinical potential of using PANX1 channel inhibitors to reduce the development of distant metastases from metastatic primary tumours [74].

Opposing trends involving Panx1 expression and cancer have also been reported in the literature. Panx1 levels are significantly reduced in gallbladder adenocarcinoma in comparison to normal gallbladder epithelium [75]. In this case, Panx1 levels negatively correlate to the proliferative activity of the cells. Additionally, Panx1 and Panx3 levels are significantly reduced in the tumour cores of basal and squamous cell carcinomas in comparison to normal epidermis [21]. Finally, a tumour-suppressive role has been noted in rat C6 glioma cells, as over expression of Panx1 was found to decrease proliferation, cell motility, anchorage-independent growth and tumorigenicity *in vivo* [76]. Interestingly, these authors also examined four human glioma cell lines (U87, U251, SF188 and SF539) and found positive PANX1 expression at the transcript level, suggesting that the role of PANX1 may differ within the same cancer type depending on the species being assessed [76].

It is clear from these reports that Panx1 expression may be regulated differently depending on the tissue, cell line, or species it is expressed in. Although human pannexins have approximately 94% conserved sequence homology to mouse pannexins, reports have specifically noted the difficulty in interpreting Panx1 comparisons between cell types from different species [9, 69]. Therefore, investigations are required to determine if the role of PANX1 in human cancer cells is similar to what has been previously identified using rodent cancer cell lines as models.

1.6 Pannexin 1 Channel Blockers

Several FDA and Health Canada approved compounds that were originally developed for other purposes have been extensively utilized in the literature to effectively block Panx1 channel function. Carbenoxolone (CBX) has been used to treat peptic ulcers and can discriminate and block Panx1 channels over connexin hemichannels at concentration of 100μM or less [38, 77]. Probenecid (PBN) has been widely used for the treatment of gout and can preferentially target and block Panx1 channels [78]. Interestingly, PBN has been

shown to have a synergistic interaction with chemotherapeutic agents [79]; however, further research is required to understand the mechanism behind this effect. Other PANX1 channel blockers include a quinolone antibiotic called Trovafloxacin (Trova) and an anti-malarial drug known as mefloquine (MFQ) [80, 81]. Each compound has hydrophobic extensions and is expected to target PANX1 channels located both intracellularly and at the cell surface [82]. In addition, each channel inhibitor has been found to attenuate Panx1-mediated release of ATP and other metabolites [14, 74, 80, 83, 84].

Chemical inhibitors of Panx1 have been shown to be effective *in vivo* at reducing disease properties with no reported significant side effects. In particular, CBX treatment in mice significantly reduced breast cancer metastases in the lungs [74]. However, the mechanism involving the interaction of these compounds on Panx1 channels has not been well defined [36, 78, 82]. Recently, a study has suggested that CBX and other inhibitors including PBN may modulate amino acids located within the first extracellular loop of Panx1 to cause an inhibitory effect by modulating the channels gating machinery rather than through simple steric blockade [36]. Since these chemicals were designed for other purposes, they may exhibit off-target effects. Therefore, the pannexin 1 mimetic inhibitory peptide, ^{10}Panx1 (WRQAAFVDSY), and monoclonal anti-Panx1 antibodies have also been used to more specifically target and block Panx1 channels [85, 86]. It may therefore be possible to utilize compounds reported in the literature to effectively block PANX1 channels as an alternative therapy in disease states where PANX1 is dysregulated.

1.7 Melanoma

Although melanoma only accounts for 5% of all skin cancer cases, it is a highly aggressive cancer and is responsible for more than 75% of skin cancer deaths [87, 88]. The incidence of cutaneous melanoma in Canada is suggested to be rising, with approximately 6800 new cases predicted in 2015 [89]. Melanoma develops from melanocytes, which are located at distinct sites throughout the body; however, 95% of all melanomas develop from skin melanocytes [58, 90-92]. Due to potential differences in

the tumour microenvironment, melanomas that differ in origin can vary in genetic mutations and histopathological presentation [93]. Multiple factors have been identified to influence the development of different melanomas including UV-exposure, a person's age and oncogenic mutations [66, 94]. In Caucasians, the most prevalent type of melanomas are derived from skin commonly exposed to the sun and can be classified as chronically sun damaged (CSD) or non-chronically sun damaged (non-CSD) [66]. CSD melanoma tumours have a high mutation burden and occur anatomically where the highest level of sun exposure occurs (e.g., head and neck) [58, 95].

There are different types of melanocytic neoplasms. A benign nevus is composed of non-cancerous, proliferating melanocytes and are commonly referred to as a mole [58]. As UV-induced point mutations are acquired, melanocytic neoplasms become polyclonal in nature and show increased levels of proliferation. Here, the cluster of melanocytes can be classified as a dysplastic neoplasm (atypical mole), which is associated with an increased risk for melanoma development [96]. Melanoma can be clinically staged into 5 distinct categories based on tumour thickness, metastasis to lymph nodes and the presence of distant metastases [97]. Melanoma *in situ* (stage 0) is used to describe melanocytes that show high proliferation levels, enlarged nuclei and irregular radial growth in the epidermis [58]. This stage has a high frequency in mutations of the MAPK (mitogen-activated protein kinase) signaling pathway including *BRAF* (encodes B-raf protein), *NF1* (neurofibromin 1; encodes a tumour suppressor protein that targets Ras), and *NRAS* (encodes N-Ras protein) [66, 98]. Melanoma is highly treatable if caught early in this stage of progression. Stage 1 and 2 of melanoma progression consists of a localized primary tumour approximately 1mm-4mm in size with or without ulceration [97]. A tumour becomes invasive once it displays vertical growth and penetrates into the dermis and the subcutaneous tissue [97]. Melanoma is classified as a metastatic disease once cells have left the primary tumour and its surrounding environment and develop in other tissues [99]. The melanoma cells will first metastasize to regional lymph nodes (stage 3) before travelling through the lymphatic and vascular system to invade distant organs (stage 4) [99]. The four most common sites for distant melanoma metastases include the lungs, brain, skin and liver [100]. In addition, it is possible for metastases in the lymph nodes and distant sites to be found when there is no detection of a primary tumour. These

are classified as melanomas of unknown primary (MUP) and have been suggested to originate from melanocytes in the skin [58, 101].

There are multiple mutations contributing to the development or progression of melanoma. *BRAF* gain-of-function mutations (V600E, V600K, V600R) are found in approximately 50-60% of melanomas and are typically identified in younger patients with variable sun-exposure, whereas the *NRAS* gene is mutated in approximately 20% of melanomas and is mainly activated in nodular and CSD primary melanomas [94, 102-104]. In addition, the *NF1* gene was found to be mutated in approximately 14% of melanoma tumour samples [103]. Collectively, these mutations activate downstream MAPK and PI3K (phosphoinositide 3-kinase) signaling pathways to initiate melanoma development and lead to changes in cell proliferation and apoptosis [58, 103, 105]. Other common melanoma mutations occur in *PTEN* (phosphatase and tensin homolog) and *KIT* genes and are associated with growth and metabolism, and *TP53* mutations are associated with a resistance to apoptosis in advanced melanoma progression [58]. In addition, *MITF* is a transcription factor known to regulate different genes that modify development, differentiation, survival, cell-cycle regulation and pigmentation in melanocytes [106]. *MITF* amplification has been identified in 20% of metastatic melanomas and may function as a ‘lineage-survival’ oncogene [56, 107].

Due to variability in melanoma development and disease progression, there is a need to better understand the underlying molecular pathways involved, so effective therapeutic alternatives can be developed.

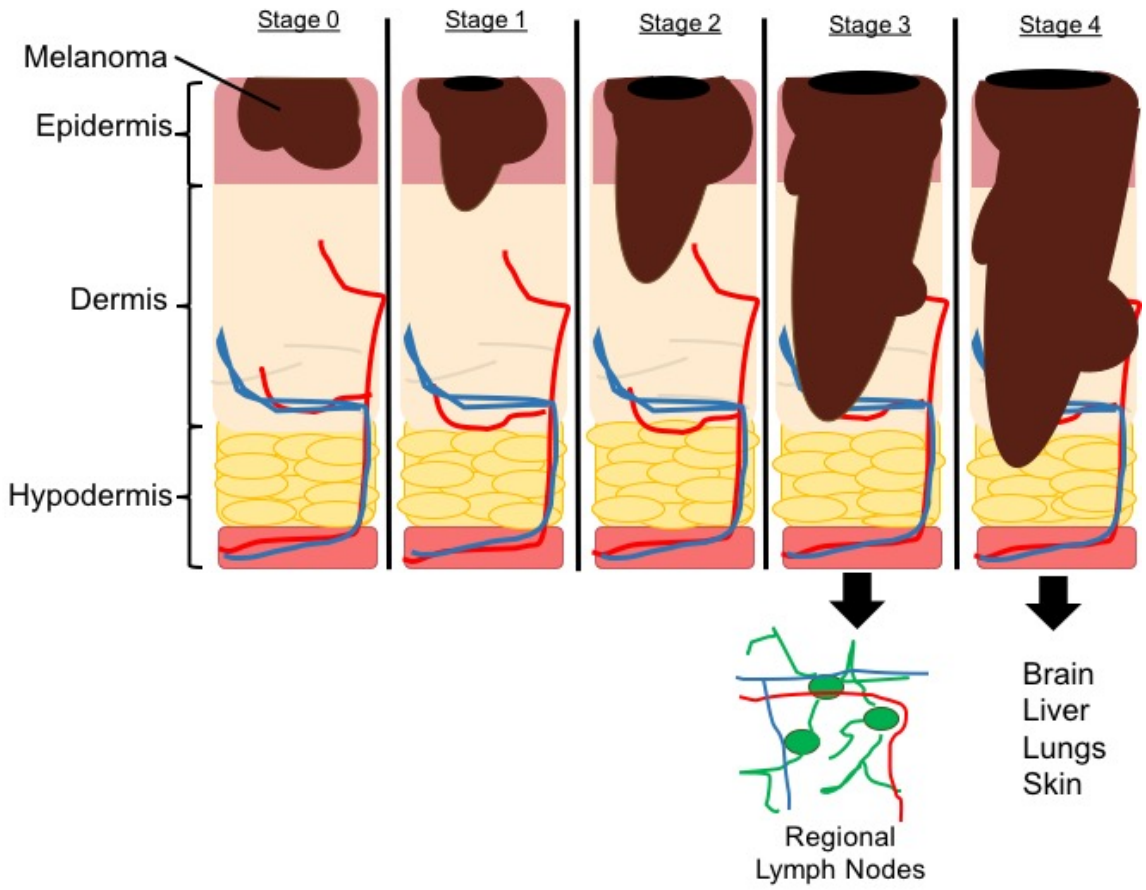

Figure 1.3. Malignant melanoma staging using TNM classification. Stage 0: melanoma *in situ*; melanoma is contained within the epidermis. Stage 1: Melanoma is ≤1mm in thickness, with or without ulceration, and invasive without metastasis. Stage 2: Melanoma is 1mm-4mm in thickness, with or without ulceration, and invasive without metastasis. Stage 3: Melanoma is invasive, with or without ulceration, and shows metastasis to 1-3 regional lymph nodes. Stage 4: Metastatic melanoma to subcutaneous tissue, lymph nodes, skin, or visceral organs.

1.8 Experimental Melanoma Models

Melanoma is a complex disease. It is therefore essential to select the most appropriate model to better understand the mechanisms involved in its progression. Since there are several differences between an *in vivo* tumour microenvironment and cell culture, it is possible that immortalized cell lines may differ from the tumours that they were established from. A study performed by Vincent and Postovit (2017) compared the transcriptome of approximately 42 melanoma cell lines to hundreds of tumours from The Cancer Genome Atlas [108]. The majority of melanoma cell lines assessed appear to be representative of patient tumours on a transcriptional level, although variations were noted in immune-related genes. The top four melanoma cell lines were determined based on the average Pearson's correlation of their gene expression profiles with single malignant melanoma cells and include COLO849, SK-MEL-30, UACC-257, and A375 [108]. The A375 melanoma cell line is derived from a primary skin melanoma lesion of a 54-year-old woman and carries mutant *BRAF* and *CDKN2A* [109]. Interestingly, the A375 cell line contained a predominantly *AXL* gene program (Axl receptor tyrosine kinase; resistance to different targeted therapies) over the proliferative *MITF* program [108, 110]. In addition, cell lines have been derived to model certain stages and phenotypes of cancer including increased metastasis. For example, the 131/4-5B1 and A375-MA2 cells were developed from diffuse lung and brain metastases of a parental human melanoma cell line and may spontaneously metastasize to the brain and lungs *in vivo*, respectively [111, 112]. Furthermore, the chick-CAM assay is an *in vivo* experimental model commonly used in cancer studies due to favourable tumour grafting on the chorioallantoic membrane and ability to study several properties of cancer including tumour growth and invasion, angiogenesis and metastasis [67, 113, 114]. Previous studies have utilized this method to analyzed tumorigenic and metastatic properties of both human and murine melanoma cells including the A375, C8161, and B16-BL6 [67, 109, 115]. Therefore, by utilizing the A375 and A375-MA2 melanoma cell lines and the chicken embryo model we may be able to effectively evaluate the role of PANX1 in several tumorigenic properties of human melanomas.

1.9 Current Treatments for Melanoma

Melanoma can be treated effectively if identified during the early stages of development. However, treatment options become limited once melanoma is classified as an invasive or metastatic disease [103]. Standard treatment for localized melanoma involves surgical resection of the tumour and biopsy of sentinel nodes (prognostic factor) [94, 116]. In the majority of cases, this approach is highly effective at treating melanoma *in situ* [58]. Radiation therapy and chemotherapy have additionally been used to treat early and advanced stages of melanoma, although these approaches show limited effects, can be cytotoxic to normal cells and do not significantly extend the survival of patients [117].

More recently, targeted therapies have been utilized to inhibit commonly mutated signal transduction molecules including *BRAF/MEK/ERK/c-KIT* [118-122]. Improved patient survival has been found in preliminary studies that used BRAF inhibitors including Vemurafenib to treat melanoma tumours [123]. By combining BRAF and MEK inhibitors, researchers found that this effect was enhanced [124]. Targeted approaches may also inhibit transcription factors in pathways downstream of the mutation. For example, where NRAS mutations are present, most strategies focus on targeting downstream of the MAPK pathway rather than targeting NRAS directly [104]. Although targeted approaches are promising therapies, most do not show long-term effects and many tumours will acquire resistance to the applied therapy [104].

In comparison to therapies which target certain proteins, enzymes or molecules involved in malignant cell growth and invasion, immunotherapies can treat cancer cells indirectly by enhancing the anti-tumour response [125]. In particular, Ipilimumab is a recombinant, human monoclonal antibody that blocks the inhibitory function of CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) on autoimmunity [116]. In doing so, Ipilimumab promotes the body's antitumour T-cell response and allows our immune system to target tumour cells. Additional immunotherapies including pembrolizumab function to promote an anti-tumour response by targeting the PD-1 ligands that inhibit T cell function [125].

At this point in time, combination therapy appears to be the best and most successful approach to treat malignant melanoma [94, 126]. Although results depend on the treatment applied and individual patient factors, preliminary results from targeted treatments and immunotherapies have shown to limit tumour progression and extend patient survival by several months [127]. Few patients show durable disease control over multiple years [128]. However, therapy resistance, lack of reliable biomarkers, cost, toxicity and risk of immune-related adverse reactions are still some of the major factors limiting the effectiveness of both therapeutic approaches [116, 129-131]. Therefore, a better understanding of molecular alteration in melanoma is required to identify novel targets for therapy that may be used in combination with current treatment options to enhance patient survival and quality of life.

1.10 Pannexin 1 in Melanoma

Panx1 has recently been highlighted as a novel target for melanoma therapy. Research conducted by Penuela et al. (2012) identified high Panx1 levels in mouse isogenic melanoma cell lines (B16-F0, -F10, -BL6) in comparison to the L10 normal melanocyte cell line (L10BIOBR-GFP) [67]. Previously, the isogenic cell lines were derived from a B16 melanoma cell line established from C57BL/6 mouse melanoma [132]. B16-F10 cells were derived from 10 successive selections for lung metastases following intravenous injection of the cells [133]. In comparison, the B16-BL6 cells were created from B16-F10 cells that metastasized to the bladder membrane and are considered to be the more aggressive cell line due to their ability to spontaneously metastasize following intraperitoneal injections [132, 134]. Interestingly, levels of Panx1 expressed in these cell lines were found to positively correlate with a more aggressive phenotype [67]. In order to determine how Panx1 was involved in the malignant properties of melanoma, researchers applied a Panx1 shRNA knockdown to BL6 melanoma cells that reduced Panx1 levels by approximately 85%. With this method, members of our research group were able to revert the malignant cells back into a melanocytic-like counterpart, which was determined both phenotypically and via an increase in melanin production [67]. In addition, Panx1-depleted BL6 cells had reduced migration, proliferation and expression of malignancy markers including β-catenin and vimentin. Furthermore, an *in vivo* chick-

CAM (chicken chorioallantoic membrane) assay was used in this study to evaluate changes in tumorigenicity and spontaneous metastasis. Tumours derived from Panx1-depleted BL6 cells weighed significantly less than control tumours. In addition, spontaneous metastasis was evaluated via qPCR analysis of genomic DNA from the chick's lungs, liver and brain using primers specific for mouse *Alu* repeats [135]. Results indicated a significant reduction in spontaneous metastasis to the liver of the chicken embryo models inoculated with Panx1-knockdown cells [67]. Taken together, this work was the first to highlight the role of Panx1 in the tumorigenic and metastatic properties of melanoma.

The Human Protein Atlas has reported high levels of PANX1 protein in approximately 70% of human melanoma tumours. In addition, the Cancer Genome Atlas (TCGA; cBioPortal for Cancer Genomics [136, 137]) has identified six somatic mutations (Q264*, T176I, H190Y, S239L, G168E, Y150F) on the *PANX1* gene from a subset of patient-derived melanoma tumours. These mutations are localized to the intracellular loop and the second extracellular loop of PANX1, and it is possible that they may affect the channel localization or function of PANX1 in melanoma cells [9]. Although these mutations have yet to be characterized, it is possible that they enhance PANX1 channel function and increase a malignant phenotype in a similar manner to mutations found in PANX1 of metastatic breast cancer cells [74].

Furthermore, the link between Panx1, purinergic signaling, melanoma and inflammation has recently been suggested [138]. A model was proposed where ATP is released from cells into the microenvironment thereby promoting the formation of PANX1/P2X7 channel complexes in melanocytes containing mutations [138]. The channel complex allows cation exchange, which activates the NLRP3 inflammasome (composed of NLR, ASC, and caspase-1) leading to direct or indirect release of the IL-1β (pro-inflammatory cytokine), prostaglandin-E2 and cyclooxygenase-2 from the tumour cells *in vitro* [138]. Once excreted into the tumour microenvironment, these factors can increase tumour growth and invasiveness, angiogenesis, cellular proliferation and pro-inflammatory cell recruitment to the tumour. [139, 140]. Further insight is required to determine the direct role of PANX1 and purinergic signaling in melanoma progression *in vivo.*

Based on the current data, it is clear that the role of PANX1 may be important in the development or progression of melanoma tumours. Therefore, further *in vitro* and *in vivo* studies should be conducted to assess the role of PANX1 in the tumorigenic properties of human melanomas and determine if it could be targeted for therapeutic intervention.

1.11 Rationale and Hypothesis

Melanoma is a highly aggressive form of skin cancer that has limited available treatment options. Panx1 is a channel-forming glycoprotein that is expressed in the majority of tissues and has been detected in both normal and disease states. Current reports have shown Panx1 involvement in normal skin development and suggest Panx1 dysregulation in skin cancers. In particular, high Panx1 levels were reported to modulate the malignant properties of mouse melanoma cell lines highlighting its potential as a novel target for melanoma therapy. Since Panx1 channel blockers have been utilized in previous reports to limit the progression of different disease states, we suspect that some of these blockers may be applied to human melanomas in order to reduce its malignant properties. Therefore, I hypothesize that PANX1 is present at each stage of melanoma progression and can be targeted with PANX1 channel blockers to reduce tumorigenic properties of human melanomas.

1.12 Objectives

i. Quantify PANX1 levels in patient-derived melanomas.

ii. Assess PANX1 involvement in the tumorigenic properties of human melanoma and its potential as a target for melanoma therapy.

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Chapter 2

2 Manuscript

Inhibition of Pannexin 1 reduces tumorigenic properties of human melanoma

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2.1 Abstract

Pannexin 1 **(**PANX1) is a channel-forming glycoprotein expressed in many mammalian organs and tissues including the skin. Pannexin 1 channels allow the passage of important signaling molecules up to 1 kDa, including ATP and other metabolites. Increased Panx1 levels were previously found to influence both tumour size and metastatic potential of mouse melanoma cell lines. In this study, we discovered high levels of endogenous PANX1 protein across multiple established human melanoma cell lines (A375-MA2, A375, A2058, C8161, 131/4-5B1), melanoma tumour biopsies, and patient-derived primary cells from each stage of melanoma progression. Western blot and immunohistochemistry data show variability in levels of PANX1 between patient samples and expression that is localized primarily to the core region of the tumours. Two PANX1 channel blockers, Carbenoxolone (CBX) and Probenecid (PBN), were applied to isogenic human melanoma cell lines in order to assess the involvement of PANX1 in both cellular and tumorigenic properties. CBX and PBN significantly reduced A375 and A375-MA2 cell growth and cell migration and significantly increased the production of melanin in both cell lines, suggesting a reversion to a more melanocytic-like phenotype. In addition, daily treatment of CBX or PBN onto A375-MA2 cells grown on the chorioallantoic membrane of a chicken embryo model significantly reduced melanoma tumour weight. Collectively, our findings suggest a possible dysregulation of PANX1 in human melanoma that may contribute to their tumorigenic properties, highlighting PANX1 as a target for melanoma therapy.

2.2 Introduction

Pannexins (Panx1, 2, 3) are a family of glycoproteins that oligomerize to form large-pore channels in the cell membrane [1-3]. Pannexin channels play an important role in allowing the passage of molecules up to 1 kDa that are important for paracrine and autocrine signaling, including adenosine triphosphate (ATP) [1-3]. One of the more extensively studied roles of Pannexin 1 (Panx1 in rodents, or PANX1 in humans) includes ATP-release for the purpose of purinergic signaling [4-8]. In particular, caspase-induced Panx1 channel release of ATP from apoptotic cells has been shown to attract

immune cells and promotes cell clearance [6]. Another important post-translational modification of pannexins includes glycosylation. N-linked glycosylation has been found to regulate channel localization, pannexin intermixing and functional status of pannexin channels [9, 10]. Panx1 is identified as a multi-banding pattern upon Western blot analyses due to the presence of its distinct glycosylation species. The highly glycosylated species of Panx1 (Gly2) has been shown to preferentially traffic to the cell surface, whereas the unglycosylated species (Gly0) and high mannose species of Panx1 (Gly1) favor intracellular locations including the ER [9]. Studies have described the presence of Panx1 channels in multiple organs including the heart, liver, brain, spleen, lung, and the skin [10, 11]. It is therefore possible that the function of Panx1 may differ between tissue types, at least in part due to variable degrees of glycosylation [10].

In addition to participating in taste sensation, airway defense, immune function and differentiation of multiple cells including those of the skin, PANX1 has also been known to participate both directly and indirectly in multiple disease states, including cancer [2, 12-15]. For most cancer types, Panx1 expression positively correlates with the onset or progression of the disease [15-18]. For example, human leukemic lymphocytes show higher levels of PANX1 in comparison to normal T cells, and over-expression of PANX1 has been reported across highly aggressive multiple myeloma cell lines [18, 19]. Furthermore, a significant reduction in proliferation of U87-MG human glioma cells was noted when a PANX1 siRNA was introduced [17]. Another group was able to show PANX1 mRNA expression in multiple human glioma cell lines; however, the authors also described a reduction in several cancerous properties of rat C6 glioma cells stably overexpressing EGFP-tagged Panx1, suggesting that opposing trends may also occur and that the role of Panx1 may differ between species [20]. Reduced levels of PANX1 and PANX3 in basal cell and squamous cell carcinomas compared to normal epidermal tissue further supports the idea that the role of PANX1 may also differ between cancer subtypes [21]. Mutated forms of the PANX1 channel was additionally reported to increase the metastatic potential of several aggressive breast cancer cell lines [4]. Co-expression of a truncated, mutated form of PANX1 ($PANX1^{1-89}$) with a full-length form of the protein in metastatic breast cancer cells led to augmented ATP release from the channel that acts on P2Y-purinergic receptors resulting in a resistance to apoptosis and enhanced cell survival

in distant organs [4]. Therefore, it is possible that PANX1 dysregulation can contribute to a malignant phenotype although its role may differ between cancer subtypes [15].

Although melanoma accounts for only 5% of all skin cancers, it is a highly aggressive disease that is responsible for more than 75% of skin cancer deaths [22, 23]. A primary melanoma tumour develops from the malignant transformation of melanocytes due to an accumulation of DNA mutations [24, 25]. Cells from the primary tumour will first metastasize through lymphatic vessels forming nodal tumour metastases and further spread throughout the vascular system to metastasize in distant organs including the brain, liver and lungs [24, 26]. Multiple melanoma cell lines, including 131/4-5B1 [27] and A375-MA2 cells [28], have been used to model cancer and its distinct phenotypes including metastasis. Additionally, the A375 melanoma cell line has been found to be highly representative of actual patient tumours on a transcriptional level, supporting its use as an effective model for melanoma [29]. Current treatments for melanoma are still limited, can result in patients developing a resistance to therapy [30, 31] and focus primarily on inhibiting proteins of altered genetic pathways that are present in a subset of the patients (i.e. BRAF) [32-34]. Despite recent advances using checkpoint inhibitors in immunotherapy [35, 36], melanoma patient survival is still limited and resistance is a prevalent outcome [37]. Therefore, a better understanding of the cellular mechanisms that drive melanoma progression is required to find additional therapeutic alternatives. Research conducted by members of our group had previously highlighted Panx1 as a possible new candidate for melanoma therapy [38]. In this study, Panx1 expression was found to be significantly up-regulated in B16-BL6 mouse melanoma cells compared to murine skin melanocytes. By using shRNA knockdown to reduce Panx1 levels in B16-BL6 melanoma cells, researchers observed a significant reduction in the malignant properties of melanoma including proliferation, cell migration, and decreased tumour size and spontaneous metastasis to the liver in a chick embryo model (chicken chorioallantoic membrane assay, chick-CAM assay) [38].

Two FDA- and Health Canada-approved broad spectrum drugs, Carbenoxolone (CBX) and Probenecid (PBN), are commonly used to block Panx1 channels and attenuate release of ATP and other metabolites [4, 6, 39]. Probenecid and Carbenoxolone have been used

for the treatment of gout [40] and peptic ulcers [41], respectively. Although their mechanism of action is not well defined, both channel blockers have been shown to target Panx1 and reduce pathological phenotypes including neurodegeneration after ischemic injury and irregular gut motility in inflammatory bowel diseases [40, 42-46]. In addition, pre-treatment with CBX significantly reduced the ability of highly aggressive breast cancer cells to metastasize to the lungs in a mouse model [4]. This treatment was well tolerated in the mice, indicating the clinical potential of CBX-based therapy to reduce the development of distant metastases [4]. It is therefore possible that PANX1 channel blockers could also be used to reduce the malignant characteristics of human melanomas.

This research is the first to identify endogenous PANX1 expression in human melanoma cell lines, patient melanoma biopsies and patient-derived primary melanoma cells. By using CBX and PBN, we were able to reduce *in vitro* cellular properties of human melanoma cells, including cell growth and cell migration, and *in vivo* tumour growth on the chorioallantoic membrane (CAM) of a chicken embryo model. These results suggest that high levels of PANX1 found in human melanomas may be associated with their malignant behaviour, further supporting PANX1 as a novel target for therapy.

2.3 Results

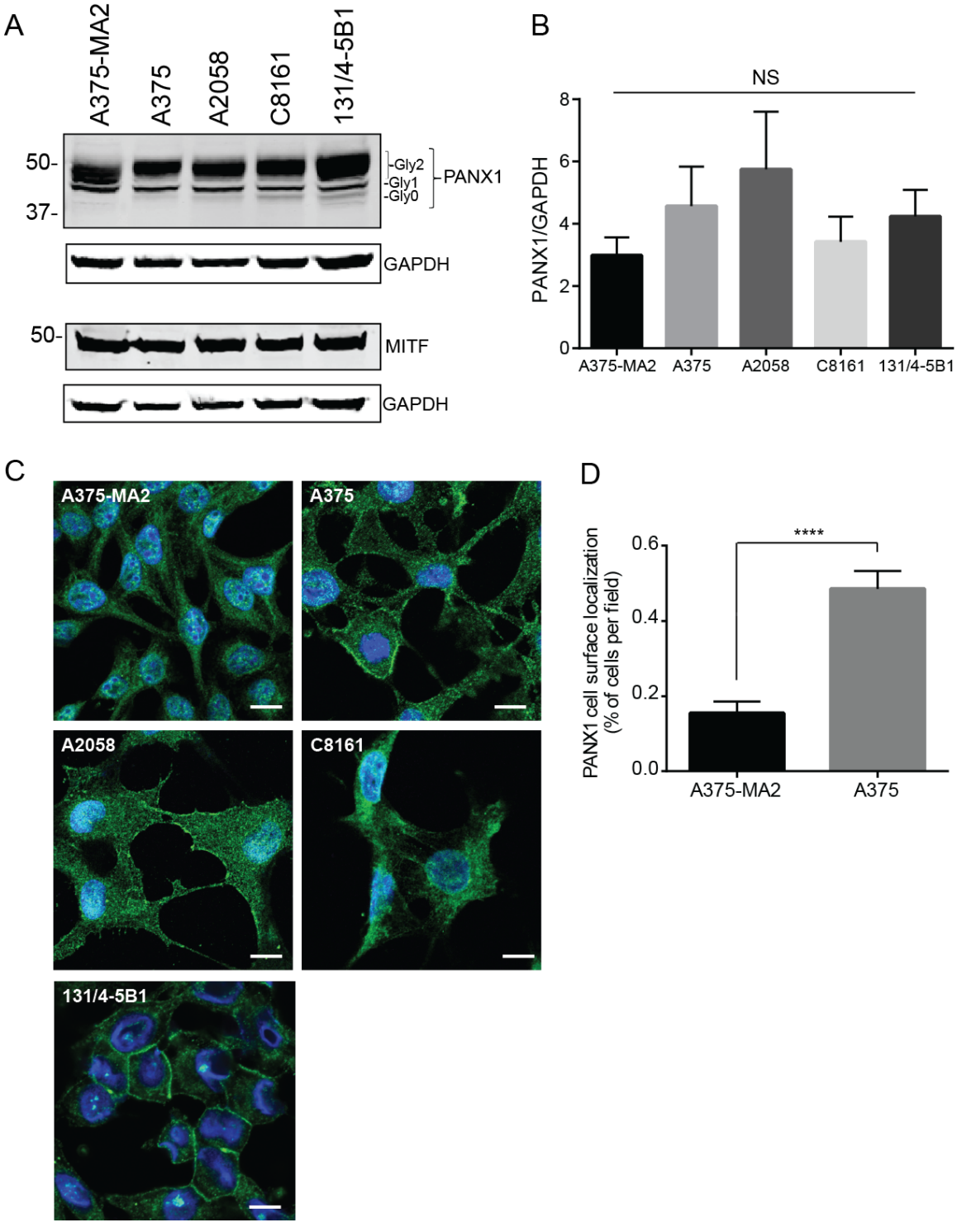
2.3.1 Pannexin 1 is expressed in human melanoma cell lines.

Panx1 expression was previously found to correlate with an aggressive phenotype in mouse melanoma cell lines [38]; however, expression of PANX1 in human melanoma is still unknown. We therefore set out to evaluate endogenous PANX1 expression in a panel of human melanoma cell lines that differ in origin and metastatic profiles. The aggressive A375 melanoma cell line was previously isolated from a primary tumour and shows transcriptional similarities to patient melanoma tumours [29, 47]. A375-MA2 melanoma cells are the isogenic cell line to A375 cells and were derived from two selections of A375 lung metastases in immunodeficient mice [28]. C8161 melanoma cells are a spontaneously metastatic cell line [48], and the A2058 melanoma cell line is reported by the American Type Culture Collection (ATCC) to be derived from lymph node metastases. Finally, the 131/4-5B1 cells were derived from selections of parental

WM239A melanoma cell line metastases to the lung and brain in immunodeficient mice and can spontaneously metastasize to these organs *in vivo* [27]. Western blot analysis of lysate extracted from A375-MA2, A375, A2058, C8161, 131/4-5B1 human melanoma cells indicate high levels of endogenous PANX1, which presents as a multi-banding pattern that is indicative of both glycosylated and un-glycosylated species of the protein [9, 10] (Fig. 1A). Although these cells vary in origin and metastatic potential, there is no significant difference in the levels of PANX1 between the cell lines (Fig. 1B). Immunofluorescence analysis reveals PANX1 channels localized both intracellularly and at the cell surface of each human melanoma cell line, consistent with the localization patterns of ectopically expressed Panx1 in N2A and 293T cells from previous reports [9, 38](Fig. 1C).

2.3.2 Cell surface localization of PANX1 is higher in A375 melanoma cells compared to A375-MA2 cells.

To assess the role of PANX1 in the tumorigenic properties of human melanomas, the A375 and A375-MA2 isogenic cell lines were selected for further analyses. Both cell lines show equivalent levels of PANX1 expression but differ in their metastatic potential (Fig. 1B). Interestingly, the protein banding pattern on the Western blot exhibited a consistent downward shift of the Gly2 band of PANX1 to less glycosylated species in A375-MA2 cells compared to A375 cells (Fig. 1A). This suggested that A375-MA2 cells may have a predominant intracellular localization of PANX1 since the highly glycosylated species of PANX1 has been primarily identified at the cell membrane [9]. To test this hypothesis, multiple immunofluorescence images of A375 or A375-MA2 cells were taken, and cells showing PANX1 labeling at the cell membrane were blindly quantified as a percentage from total cell number by two evaluators. Based on the appearance of cell membrane labeling, there is a significantly lower percentage of PANX1 at the cell surface of A375-MA2 cells compared to A375 cells (Fig. 1D). This suggests unique differences between the cell lines in PANX1 localization as equivalent levels of PANX1 were quantified between the A375 and A375-MA2 cells (Fig. 1B).


A
A375-MA2
A375
A2058
C8161
131/4-5B1
50-
37-
Gly2
Gly1
Gly0
PANX1
GAPDH
50-
MITF
GAPDH
B
NS
PANX1/GAPDH
8
6
4
2
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A375-MA2
A375
A2058
C8161
131/4-5B1
C
A375-MA2
A375
A2058
C8161
131/4-5B1
D
****
PANX1 cell surface localization
(% of cells per field)
0.6
0.4
0.2
0.0
A375-MA2
A375

Figure 2.1. Pannexin 1 is expressed in human melanoma cell lines. *A.* PANX1 is expressed in A375-MA2, A375, A2058, C8161, 131/4-5B1 human melanoma cell lines. Melanoma cell lines express high levels of PANX1 and are positive for a melanocytic-lineage marker, MITF. *B.* Quantification of PANX1 levels in melanoma cell lines was performed using densitometry. There is no significant difference in PANX1 between the cell lines. GAPDH was used as a loading control. Statistical analysis was performed using a one-way ANOVA followed by a Tukey's *post-hoc* test; Bars indicate s.e.m., N=3, n=3. *C.* Immunofluorescence of endogenous PANX1 expression in each human melanoma cell line. PANX1 channels are localized intracellularly and at the cell membrane. PANX1: green, Hoechst: blue; Scale: 20μm. *D.* Quantification of PANX1 localized to the cell membrane was completed by dividing the number of cells that appeared to have cell surface PANX1 expression in each field of view by the total number of cells in that field. A375 melanoma cells have significantly more PANX1 channels localized to the cell surface in comparison to A375-MA2 cells. Statistical analysis was performed using Student's t-test, ****P<0.0001; Bars indicate s.e.m., N=3.

2.3.3 PANX1 channel blockers reduce tumorigenic properties of A375 and A375-MA2 melanoma cells *in vitro.*

Several malignant properties of a highly aggressive B16-BL6 mouse melanoma cell line were reduced following an shRNA knockdown of Panx1, suggesting a potential therapeutic value in targeting Panx1 channels to decrease melanoma progression [38]. We aimed to evaluate changes in cell growth, cell migration and melanin production in A375 and A375-MA2 melanoma cell lines using two PANX1 channel blockers. Carbenoxolone and Probenecid were selected to block PANX1 channels due to their availability as Health Canada-approved compounds and common use as *bona fide* channel blockers [40, 43, 49, 50]. Based on the results from cytotoxicity assays, 100μM CBX or 1mM PBN were used for *in vitro* and *in vivo* experiments as these concentrations have been reported to effectively block Panx1 channels and did not cause cytotoxic effects in A375 and A375-MA2 melanoma cell lines (Supplemental Fig 1A, B). We first developed growth curves to assess changes in cell number when each cell line was treated with PANX1 channel blockers. Following a one-time application of 100μM CBX or 1mM PBN in serum-containing media, A375 and A375-MA2 melanoma cell growth was significantly reduced starting on day four of the experiment in comparison to the vehicle control (Fig. 2). The reduction in cell growth is most evident on day five of each experiment.

Additionally, we assessed changes in the ability of the cells to migrate using scratch wound assays. The distance A375 cells migrated three days following the initial scratch was significantly reduced when 50μM CBX or 1mM PBN in serum-free media was present (Fig. 3A). Under these conditions, A375-MA2 cells showed low levels of adherence to culture dishes even under control treatment conditions. When the protocol was modified to include 10% FBS serum media, A375-MA2 cells migrated significantly less when 100μM CBX or 1mM PBN was applied (Fig. 3B). In addition, A375 cells show greater levels of cell migration in control conditions compared to A375-MA2 cells.

Furthermore, previous reports have suggested melanin to be a marker of a melanocytic-like phenotype [38]. To evaluate changes in melanin concentration when PANX1 channel

blockers are applied, melanin was extracted from A375 or A375-MA2 cells cultured with either CBX, PBN or the vehicle control for three days. By applying 100μM CBX, A375 cells produced 8.20 μg/μL more melanin (Fig. 4A) and A375-MA2 cells produced 22.59 μg/μL more melanin (Fig. 4B). Similarly, A375 cells incubated with 1mM PBN produced 10.55 μg/μL more melanin (Fig. 4A) and A375-MA2 cells produced 21.87 μg/μL more melanin (Fig. 4B). In each set of experiments, cells produced significantly more melanin when treated with each PANX1 channel blocker. Taken together, these results suggest a reduction in cellular properties that could contribute to the malignant phenotype of human melanoma cells when PANX1 channels are blocked using CBX or PBN.

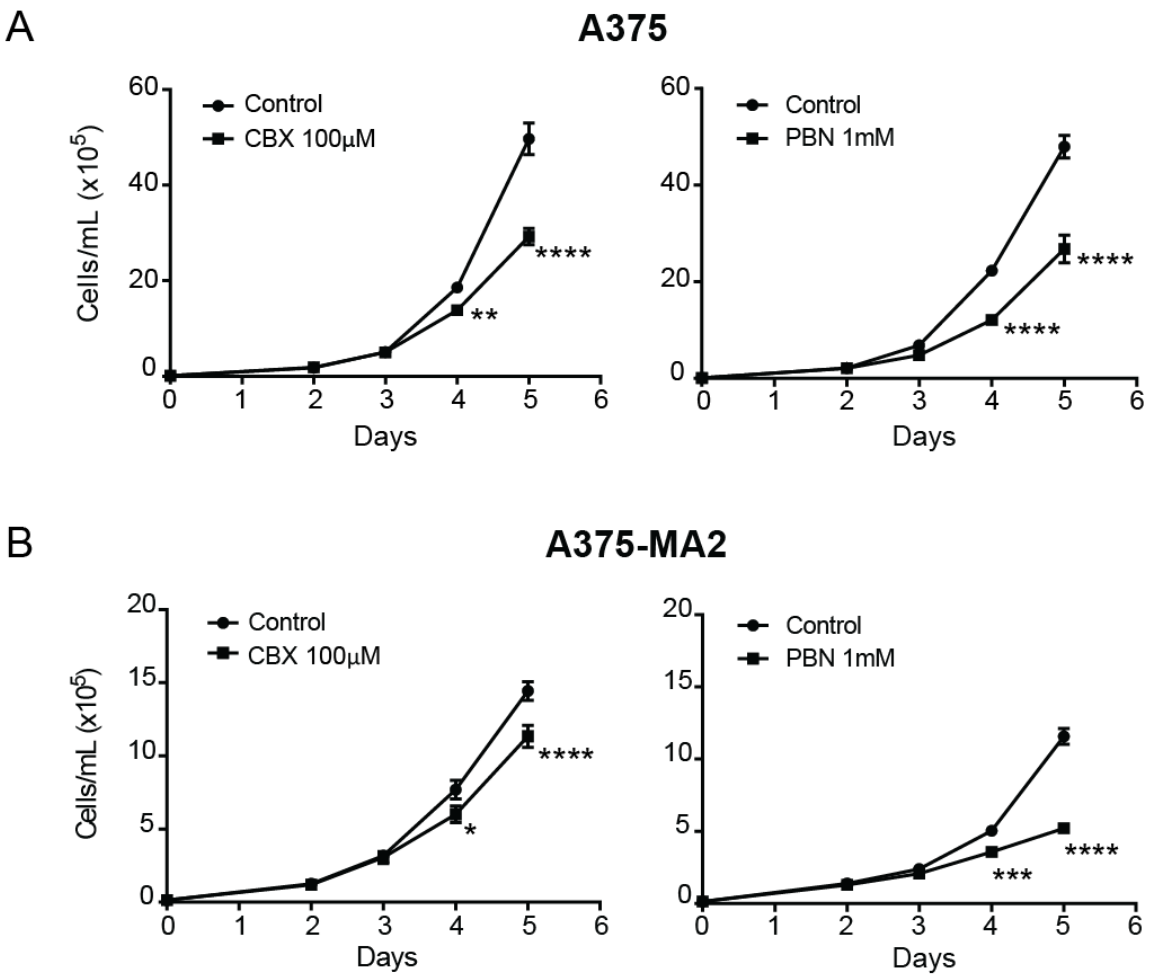


Figure 2.2. Probenecid and Carbenoxolone significantly reduce A375 and A375-MA2 cell growth. *A.* A single application of either 100μM CBX (N=3, n=9) or 1mM PBN (N=3, n=9) on A375 melanoma cells resulted in a significant decrease in cell growth on day four and five of each experiment. *B.* A375-MA2 melanoma cells also grew significantly less on day four and five when either 100μM CBX (N=4, n=12) or 1mM PBN (N=3, n=9) was applied. Two-way ANOVA with multiple comparisons followed by a Sidak test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; Bars indicate s.e.m.

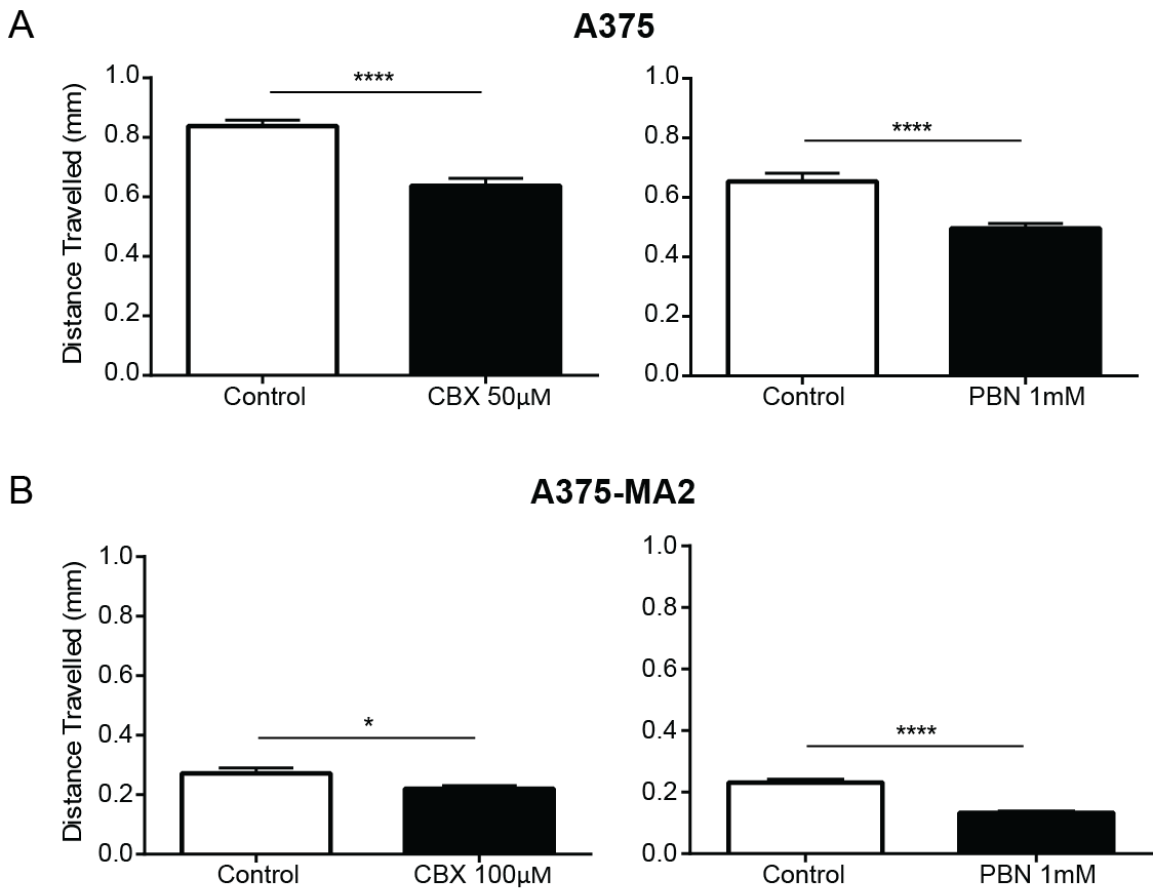


Figure 2.3. A375 and A375-MA2 cell migration is significantly reduced upon treatment with PANX1 channel blockers. *A.* Scratch assays were performed on a confluent monolayer of A375 cells to assess migration. The distance travelled by the cells represents cell migration three days following the initial scratch and treatment application. Application of 50μM CBX (N=3, n=32) or 1mM PBN (N=3, n=24) in serum-free media reduced A375 cell migration in comparison to treatment with the vehicle control. *B.* Scratch assays were modified to include serum-containing media for A375-MA2 cells. A significant decrease in A375-MA2 cell migration was noted when 100μM CBX (N=3, n=20) or 1mM PBN (N=3, n=22) were added to the culture media. Since serum was added to the procedure, it is possible that the differences are due to a combination of cell growth and migration. Statistical analysis includes a student's t-test; *P<0.05, ****P<0.0001; Bars indicate s.e.m.

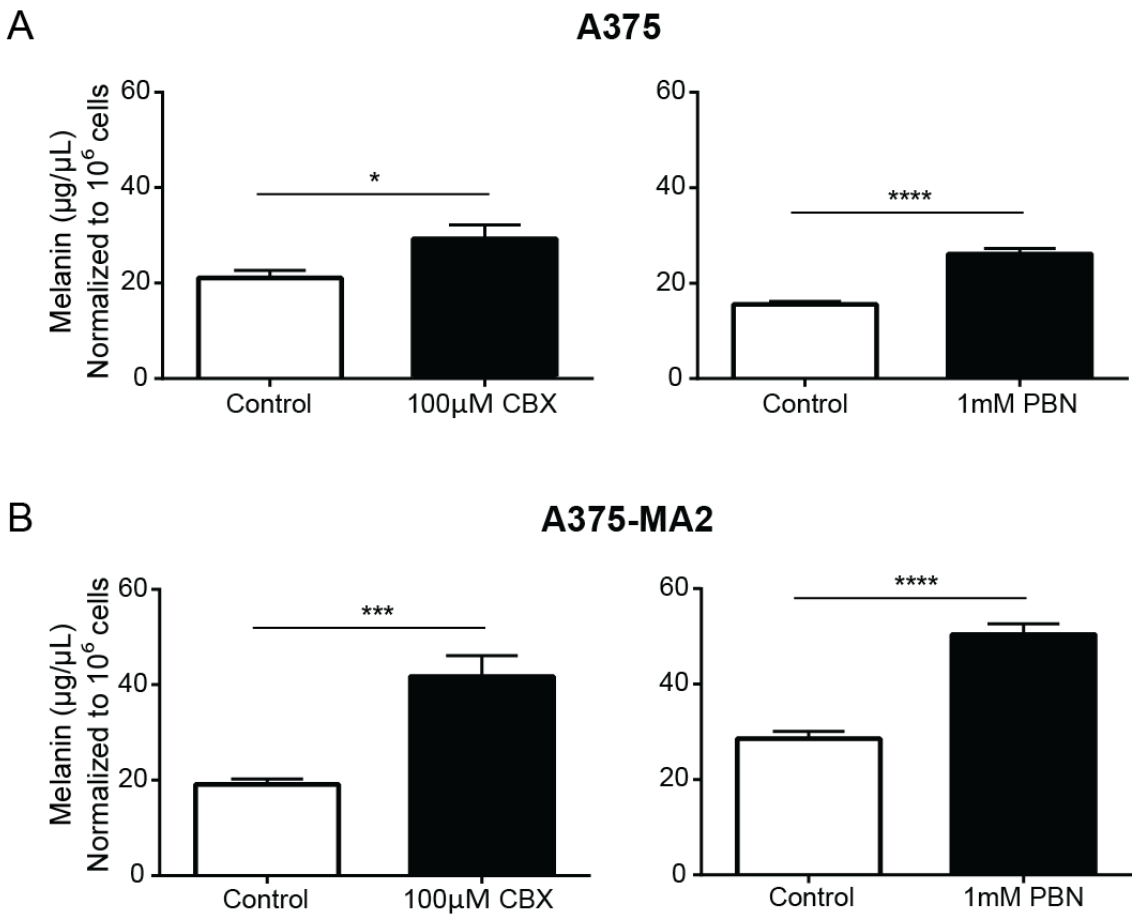
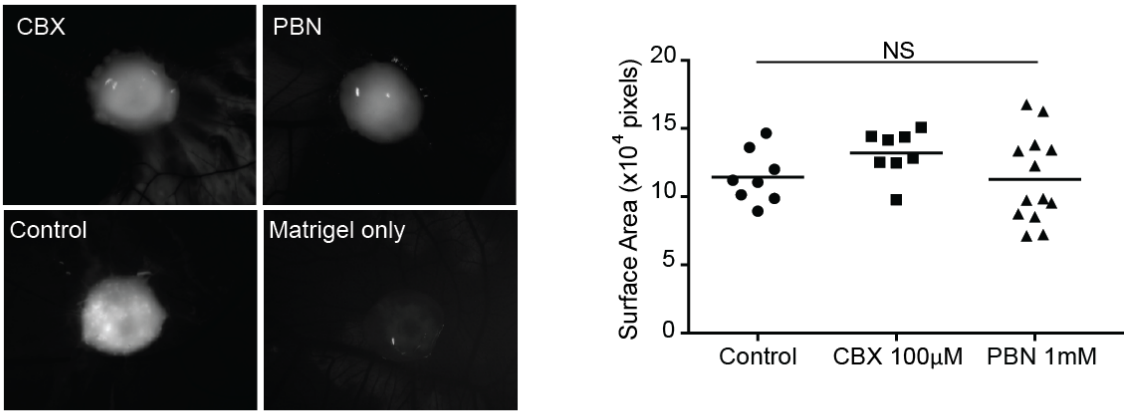


Figure 2.4. Human melanoma cells produce significantly more melanin when treated with PANX1 channel blockers. *A.* A375 melanoma cells produced significantly more melanin when 100µM CBX (N=3, n=23) or 1mM PBN (N=3, n=27) was applied, measured by the concentration of melanin (µg/µL) extracted from one million cells. *B.* Similarly, significantly more melanin was extracted from A375-MA2 melanoma cells treated with either 100µM CBX (N=3, n=15) or 1mM PBN (N=3, n=13) in comparison to the vehicle control. These results suggest that blockade of PANX1 may result in a more melanocytic-like phenotype. Statistical analysis includes a student's t-test; *P<0.05, ***P<0.001, ****P<0.0001; Bars indicate s.e.m.

2.3.4 Probenecid and Carbenoxolone significantly reduced A375-MA2 tumour growth in an *in vivo* model.

In order to evaluate changes in tumour growth when PANX1 channels are blocked, a chicken embryo model was utilized. One million A375-MA2 melanoma cells were pre-loaded with DiO lipophilic cell tracer, mixed with Matrigel and seeded onto the chorioallantoic membrane (CAM) of ten-day-old chicken embryos. PBN, CBX or a vehicle control was directly applied to visible tumours daily following the initial inoculation for six days. Tumours were visibly white in each treatment condition and appeared to grow on top of the CAM in the z-axis. There was no significant difference in tumour surface area (i.e. x- and y-axis) when PANX1 channel blockers were applied (Fig. 5A). However, when tumours were excised and weighed, we discovered that tumours treated daily with either 100μM CBX or 1mM PBN weighed significantly less than those treated with the vehicle control, possibly due to overall changes in tumour growth (Fig. 5B). These results support a reduction in tumour growth when PANX1 channel blockers are applied to human melanoma tumour xenografts in the chicken embryo model.

A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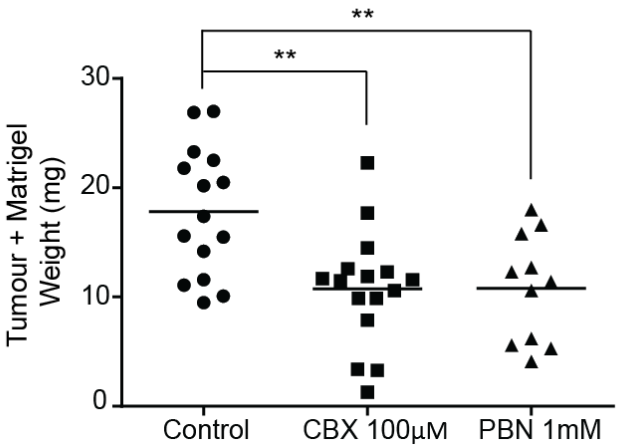
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CBX 100µM
PBN 1mM

Figure 2.5. Carbenoxolone or Probenecid significantly reduced A375-MA2 melanoma tumour weight. *A.* One million A375-MA2 human melanoma cells were incubated with a DiO'lipophilic cell tracer, combined with Matrigel (1:1) and seeded onto the CAM of a ten-day-old chick embryo *ex ovo*. Images of tumours were taken on day 18 of the experiment and measured using ImageJ software. There was no significant difference in A375-MA2 tumour surface area when treated with 100μM CBX (N=8), 1mM PBN (N=13), or a vehicle control (N=8). One-way ANOVA followed by a Tukey's *post-hoc* test. Horizontal lines indicate data mean. *B.* A375-MA2 tumours treated for one week with 100μM CBX or 1mM PBN weighed significantly less than tumours treated with the vehicle control. One-way ANOVA followed by a Tukey's *post-hoc* test, **P<0.01; Control (N=15), CBX (N=16), PBN (N=11).

2.3.5 Pannexin 1 is expressed in patient-derived primary melanoma tumours, nodal and distant melanoma metastases.

There can be considerable differences between cell culture and an *in vivo* microenvironment. Therefore, we assessed PANX1 levels in patient melanoma biopsies to determine if PANX1 was present at all stages of melanoma progression in human tumours. PANX1 protein was readily detected in primary tumours, nodal and distant metastases obtained from the Ontario Institute for Cancer Research (Fig. 6A; Table 1). All samples were positive for PANX1 expression; however, high variation in PANX1 expression was noted among patients, and no significant difference was observed in PANX1 levels between each distinct stage of melanoma development in the samples examined (Fig. 6B). Using a melanocytic-lineage marker to identify melanoma tumour cells (MITF;[51]) and sequentially sectioned biopsies stained using H&E, PANX1 expression was found primarily in melanoma tumour cores and some stromal regions suggesting specificity in its expression to viable tumour cells (Fig. 6C). Representative immunofluorescence data from primary, nodal and distant melanoma biopsies were consistent with our Western blot data by showing patient-dependent variation in PANX1 levels (Fig. 6D; Supplemental Fig 2-4).

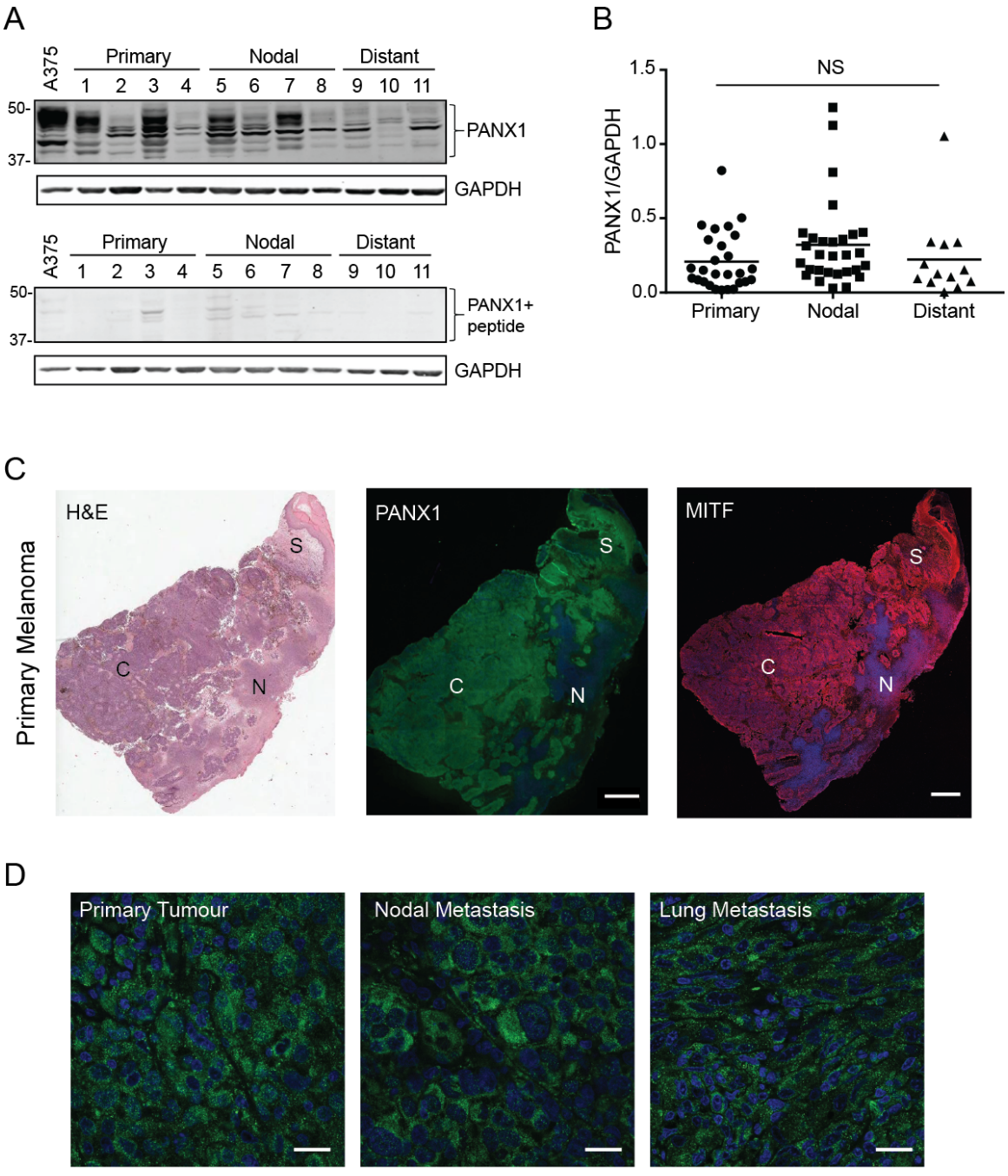
A
A375
Primary
Nodal
Distant
1 2 3 4 5 6 7 8 9 10 11
50-
37-
PANX1
GAPDH
PANX1+ peptide
B
NS
PANX1/GAPDH
1.5
1.0
0.5
0.0
Primary
Nodal
Distant
C
Primary Melanoma
H&E
PANX1
MITF
S
C
N
D
Primary Tumour
Nodal Metastasis
Lung Metastasis

Figure 2.6. Pannexin 1 is expressed in patient-derived primary melanoma tumours, nodal and distant melanoma metastases. *A.* Representative PANX1 expression in patient-derived melanoma tumours. A375 lysate was used as a positive control. A peptide competition (1:50) confirms antibody specificity to same tumour lysate. *B.* Densitometry was used to quantify PANX1 levels in all primary, nodal and distant melanoma samples provided by OICR, normalized to GADPH. There is no significant difference in PANX1 levels between each stage of melanoma progression. PANX1 expression in separate Western blots was normalized to A375 cell lysate. Primary melanoma N=6, n=27, Nodal metastasis N=7, n=29, Distant metastasis N=3, n=13; Statistical analysis includes a one-way ANOVA followed by Tukey's *post-hoc* test, horizontal line represents data mean. *C.* Patient-derived primary melanoma tumour from 70-year-old female labeled with PANX1. Sequential sections of the tumour stained using H&E (Provided by OICR) and a marker for a melanocytic-lineage, MITF. Melanoma core co-labels with PANX1 expression confirming labeling specificity. *C*- Melanoma tumour core, *N*- Necrotic region of the melanoma tumour, *S*- Stromal area of the tumour. Scale: 1000μm. *D.* Representative PANX1 expression in primary melanoma tumours (N=14), nodal melanomas (N=14), and distant melanoma metastases (N=6). PANX1 levels indicate patient dependent variation. PANX1: green, MITF: red, Hoechst: blue; Scale: 20μm.

2.3.6 PANX1 is highly expressed in patient-derived primary melanoma cells.

In collaboration with local surgical oncologists (University Hospital and Victoria Hospital, London Health Sciences Centre (LHSC), London, ON, Canada) primary cells were extracted and cultured from fresh patient-derived primary, nodal and distant melanoma tumours to more closely compare PANX1 levels and localization to established human melanoma cell lines. To assess the identity and purity of primary melanoma cell cultures, the presence of MITF was examined via Western blots and immunofluorescence microscopy (Fig. 7). Our results show high PANX1 levels in primary cells derived from each stage in melanoma progression (Fig. 7A). Localization profiles appear to be similar to results found in established human melanoma cell lines, as PANX1 channels were localized both intracellularly and at the cell surface of low-passage melanoma cells (Fig. 7B, compare with Fig. 1C). Comparable results were also obtained in a patient-matched set of primary melanoma cells extracted from a primary tumour and a nodal metastasis (Fig 7. C, D). Taken together, these limited numbers of human melanoma biopsies, patient-derived primary cells and established human melanoma cell lines show high endogenous PANX1 protein levels at all stages of melanoma progression.

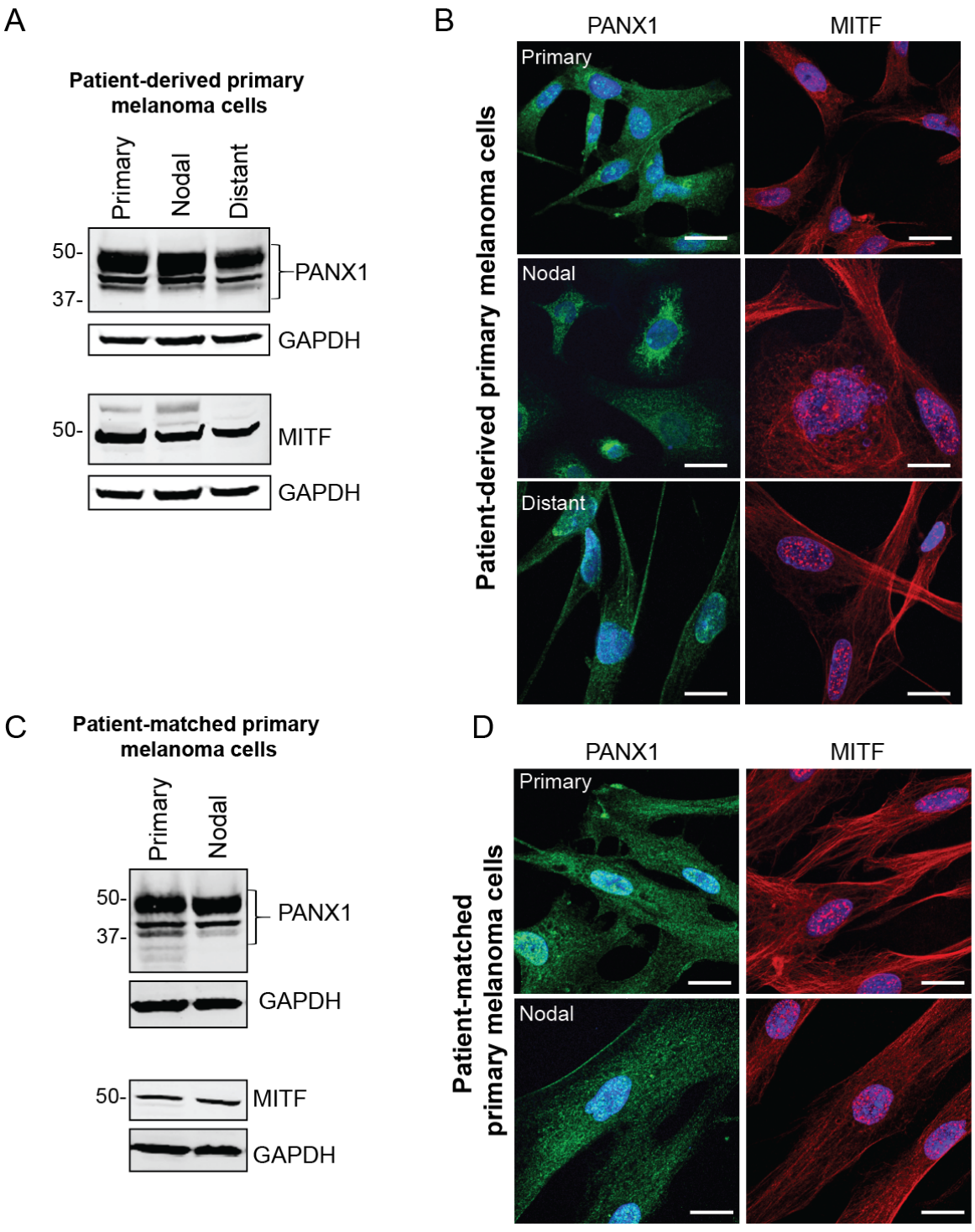
A
Patient-derived primary melanoma cells
Primary
Nodal
Distant
50-
37-
PANX1
GAPDH
50-
MITF
GAPDH
B
PANX1
MITF
Patient-derived primary melanoma cells
Primary
Nodal
Distant
C
Patient-matched primary melanoma cells
Primary
Nodal
50-
37-
PANX1
GAPDH
50-
MITF
GAPDH
D
PANX1
MITF
Patient-matched primary melanoma cells
Primary
Nodal

Figure 2.7. PANX1 is highly expressed in patient-derived primary melanoma cells. *A.* Representative PANX1 levels in primary cells derived from patient primary (N=3), nodal (N=3) and distant (N=3) melanoma biopsies. Cultures of primary melanoma cells were distinguished through MITF expression. *B.* Patient-derived primary melanoma cells extracted from the three distinct stages of melanoma progression localize PANX1 intracellularly and at the cell membrane. MITF is a transcription factor involved in melanocytic lineages and is localized in the nucleus and in the cytoplasm of the cell. PANX1: green, MITF: red, Hoechst: blue. Scale: 20μm. *C.* Patient-matched primary cells were extracted from a primary tumour and a nodal metastasis within a single patient and show high PANX1 levels. Melanoma identity was confirmed with MITF expression. Western blot provided by Danielle Johnston (Penuela Lab). *D.* Patient-matched primary cells label PANX1 in a similar manner, showing intracellularly and cell membrane localization of the channel. PANX1: green, MITF: red, Hoechst: blue; Scale: 20μm.

2.4 Discussion

The role of Pannexin 1 in both normal and disease states has been widely studied [2, 15, 52]. Panx1 expression has been found in multiple organs, including the skin, and has been shown to play an important role in keratinocyte and fibroblast differentiation [14, 53]. By analyzing Panx1 levels in dorsal skin from neonatal and adult mice, the authors suggested that Panx1 may be required during the early stages of tissue development; however, expression of this channel in adult tissue could potentially result in abnormal states [53]. Reports on inflammatory bowel diseases, ischemia and several cancers further support this hypothesis [15, 46, 54]. In most cancer studies, high Panx1 expression appears to relate to the onset or progression of disease, although this role varies depending on the species and cell analyzed [15-18]. Members of our group depleted Panx1 expression in highly aggressive mouse melanoma cells causing tumour growth and metastasis to the liver of a chicken embryo model to be attenuated [38]. Although these results suggest a potential therapeutic value in reducing Panx1 levels in melanoma, translating these results between species may not be reliable as studies investigating human and rat glioma cell lines have shown a species-dependent, tumour-enhancing or tumour-suppressive effect of Panx1, respectively [17, 20]. We therefore aimed to evaluate PANX1 levels in human melanomas and determine if blockade of the channel reduces both cellular and tumorigenic properties of human melanoma cell lines.

We have identified high endogenous PANX1 expression in each of the established human melanoma cell lines analyzed. These results coincide with the majority of cancer-related reports including human multiple myeloma cells, leukemic T lymphocytes, and mouse melanoma cells, which show dysregulated PANX1 expression in a malignant phenotype [15]. Surprisingly, PANX1 levels were equivalent between our cell lines and did not correlate with an aggressive or metastatic phenotype as previously shown in isogenic mouse melanoma cell lines [38]. In addition, reports of other skin malignancies that develop from keratinocyte cells, including basal and squamous cell carcinomas, show an opposite trend of down-regulated PANX1 within the tumour core compared to the normal epidermis [21]. Collectively, our results add to the idea that PANX1 may have a species-dependent and cell type-specific roles in different cancers.

Two aggressive, genetically similar human melanoma cell lines were selected for *in vitro* and *in vivo* experiments. A375 cells were selected based on a recent report that scored multiple melanoma cell lines on their ability to closely represent the parental tumours from which they were established [29]. These cells exhibited a fast growth rate and show resilience in cell culture. Relative to A375 cells, the A375-MA2 cells show efficient tumour growth *in vivo* but adhered less to plastic culture plates *in vitro* when serum was removed from the media for migration analyses. Interestingly, we quantified less PANX1 at the cell membrane of A375-MA2 cells. Studies have shown that the cellular distribution of pannexins may relate to the functional outcome of the channel including a role for ATP release at the cell surface and a potential role as Ca^{2+} leak channels in the ER [3, 6, 9]. Although future experiments will need to confirm these findings using cell-surface biotinylation assays, it is possible that phenotypic variations between the cell lines may be connected to PANX1 localization.

Previous reports involving human glioma or mouse melanoma cells have shown therapeutic value in using siRNA or shRNA knockdown to reduce Panx1 levels [17, 38]. When considering new therapeutic alternatives, several compounds including CBX and PBN have been shown to effectively block PANX1 channels [40, 42-46]. In this report, we provide evidence that PANX1 channel blockers can reduce cell proliferation and human melanoma tumour growth. We predict that melanoma cells containing high levels of PANX1 will release ATP or other signalling molecules to potentially act on purinergic receptors leading to increased levels of cell growth in each cell line [55]. Since cell growth was significantly reduced four days following initial application of CBX or PBN to melanoma cells and both compounds are expected to cross the cell membrane, we suspect that PANX1 may regulate molecular pathways involved in cell proliferation, potentially through purinergic signalling in conjunction with P2X7 receptors [56], that require time before changes in cell growth can be quantified. Furthermore, we identified a significant decrease in A375 cell migration when PANX1 channel blockers were applied to human melanoma cells. Since A375-MA2 migration experiments were modified to include serum media, it is possible that the results are due to a combination of cell movement and proliferation. However, there is no significant difference in A375-MA2 cell growth on day 3 when PANX1 channel blockers are applied (Fig. 2B), which

suggests that differences observed are primarily due to cell migration. Panx1 channels have been shown to interact with filamentous actin through the carboxy-terminal tail of Panx1 [57, 58]. Previous research has also identified a possible interaction between Panx1, actin and Arp3 (actin-related protein 3), a cytoskeleton-modulating protein that is apart of a complex that may indirectly contribute to the generation of actin-mediated mechanical force [57, 59]. It is therefore possible that the changes in cell migration of human melanoma cell lines may occur from direct modulation of actin by Panx1.

Changes in mole and tumour pigmentation can be used as a diagnostic tool for the progression of primary melanomas [60]. By applying PBN and CBX, a significant increase in melanin production was observed in A375 and A375-MA2 melanoma cells. High levels of melanin were similarly recorded in L10 mouse melanocytes and Panx1-knockdown BL6 melanoma cells, and have been shown to be characteristic of a more melanocytic-like phenotype [38, 61]. Transcription factors downstream of the β-catenin protein in the Wnt signaling pathway are known to regulate cell proliferation and MITF [62, 63]. MITF is a transcription factor that has been reported to regulate motility, proliferation, differentiation and pigmentation in both melanocytes and melanoma cells [64]. We examined MITF expression in all human melanoma cell lines, patient-derived melanoma biopsies and primary cells. It may therefore be possible that the changes we see in melanin production are related to the Wnt/β-catenin pathway through changes in MITF regulation. A study performed on a highly aggressive BL6 mouse melanoma cell line supports this hypothesis as β-catenin levels were noted to decrease upon Panx1 shRNA knockdown [38].

In order to determine the effectiveness of PANX1 blockers at reducing tumorigenicity *in vivo,* a chick-CAM assay was selected based on its ability to study several properties of cancer including tumour growth, cell invasion and metastasis [38, 65]. A375-MA2 cells were selected due to the reported metastatic nature of this cell line *in vivo*, and because A375 cells showed ineffective tumour growth *in vivo* despite its highly aggressive nature *in vitro* (data not shown). Tumours treated with PBN or CBX for one week weighed significantly less than tumours treated with the vehicle control, consistent with a previous report showing reduced tumorigenicity of mouse melanoma cells when Panx1 shRNA

knockdown was applied [38]. However, tumours formed in each treatment condition appeared white, which differs from reports of haemorrhagic tumours formed using B16-BL6 mouse melanoma cells [38]. Due to the fast growth rate of A375-MA2 melanoma cells and the limited incubation time on the CAM, it is possible that changes in tumour growth are primarily due to an altered cell cycle pathway [38, 66]. Taken together, these results extend our *in vitro* observations and suggest a role for PANX1 in the tumorigenicity of human melanomas.

Approximately 70% of human melanoma tumours assessed by the Human Protein Atlas show significant PANX1 expression. Similarly, we have discovered variable levels of PANX1 across each stage of melanoma progression in patient-derived melanoma biopsies and primary melanoma cells. Although patient-dependent differences should be considered, this expression suggests that PANX1 channel blockers could be applied at each stage of melanoma progression to reduce tumour growth. In addition to inhibiting PANX1 channels, PBN and CBX may exhibit off-target effects as both compounds were originally designed for other purposes [40, 67]. A recent report has suggested that CBX may modulate the first extracellular loop of Panx1 to cause an inhibitory effect, however, the mechanism involving the interaction of these compounds on PANX1 channels has not been well defined [40, 42, 43]. From our cytotoxicity assays and *in vitro* and *in vivo* analyses, both drugs appear to be well tolerated in our cell lines. This supports past work showing no significant side effects when both PANX1 channel blockers are used for short and long-term treatment regimens [4, 68]. However, future work could utilize more specific inhibitors including a ^{10}PANX1 peptide and specific anti-PANX1 monoclonal antibodies to limit possible off-target effects [69, 70].

Once melanoma progresses into a metastatic disease, the number of treatment options become limited, rarely maintain a state of remission, and may possibly lead to acquired resistance in patients [71]. Considering recent reports that suggest that cancer cells can disseminate and spread during the early evolution of primary tumour development, our need for long-term and effective treatment options is imperative [72, 73]. With this study, we are the first group to identify endogenous PANX1 expression in human melanoma cells lines and at each stage of melanoma progression in patient-derived melanoma

biopsies and primary cells. We have additionally noted a reduction in human melanoma cell growth, migration and tumorigenicity by applying two compounds that could be used as re-purposed drugs to block PANX1 channels and reduced melanoma progression and tumour growth. Collectively, this work suggests that high levels of PANX1 in human melanoma may be associated with the malignant behaviour of the cells. We also provide support for PANX1 channel blockers as an effective, alternative treatment option for melanoma that may be used in combination with current therapies in the future to increase melanoma treatment effectiveness, reduce resistance and increase patient survival.

2.5 Material and Methods

Cell Lines and Culture Conditions

Human melanoma cells lines including A2058 (ATCC CRL-11147), A375 (ATCC CRL1619) and A375-MA2 (ATCC CRL3223; [28]) were cultured in Dulbecco's Modified Eagle Medium 1X (DMEM 1X) containing 4.5g/L D-Glucose, L-Glutamine, 110mg/L Sodium Pyruvate, 5mL of penicillin-streptomycin (10,000 units penicillin, 10mg/mL streptomycin), and 10% FBS (Invitrogen). The C8161 cells (kindly provided by Dr. Lynne Postovit, University of Alberta), and 131/4-5B1 cells (a gift from Dr. Robert Kerbel, Sunnybrook Health Science Centre (Toronto, ON); [27]) were maintained in RPMI media with 10% FBS and 5mL penicillin-streptomycin. All cells were incubated at 37°C at 5.0% CO_2. Trypsin (0.25%, 1mM EDTA 1X; Life Technologies) was used to dissociate cells from culture dishes.

Pannexin 1 Channel Blockers

Carbenoxolone disodium salt (≥ 98%; Sigma Aldrich) and water soluble Probenecid (77mg/mL; Invitrogen) were dissolved in Hanks's Balanced Salt Solution (HBSS 1X, Life Technologies; calcium chloride, magnesium chloride, magnesium sulfate) to develop stock concentrations of each chemical compound.

Primary Melanoma Cells

Melanoma tumour biopsies obtained according to HSREB#103381 (Western University and LHSC), were dissected by a pathologist at University Hospital or Victoria Hospital (LHSC) and immediately placed on ice in a tube containing DPBS (Dulbecco's Phosphate Buffered Saline; calcium chloride, magnesium chloride) and 10% FBS (Fetal Bovine Serum; ThermoFisher). The tissue was processed 60-90 minutes from harvest. Samples were placed in a petri dish and washed twice with PBS to remove blood cell residue. Samples were transferred to a new petri dish and minced using sterilized scissors for 3-4 minutes. Minced tissue was then transferred to a tube containing 5mL of a freshly prepared collagenase digest solution (Krebs-Ringer bicarbonate buffer (Sigma Aldrich), 2% BSA, 50mg collagenase (Worthington Biochemical Corporation)) and digested for 30 minutes. The tissue was further separated in this mixture using sequentially smaller sterile pipettes (25mL, 10mL, 5mL) for 10-15 minutes. The final tissue suspension was filtered through a 100μm cell strainer and centrifuged at 800g x 10 minutes. Media was aspirated from the pellet and samples were re-suspended in 3mL of DMEM combined with 10% FBS, 1% non-essential amino acids (Life technologies), 2% MEM vitamin solution (Life Technologies) and 1% penicillin streptomycin. Suspended cells were plated on a sterile 10cm culture dish and incubated for 3 days at 37°C at 5.0% CO_2 to allow blood cells to separate from melanoma cells before media was replaced. Cell cultures were expanded for experimental analyses. Primary cells were fixed for immunohistochemistry or lysate was extracted from primary cells at first passage (p1).

Protein Extraction and Immunoblotting

Frozen melanoma tumour biopsies (18 total: 6 primary melanomas, 8 nodal metastases, 4 distant metastases) were obtained from the Ontario Institute for Cancer Research (OICR, HSREB#103381). Protein lysates were extracted on ice from frozen biopsy samples using a 2X IP Triton-based extraction buffer (1% Triton X-100, 150mM NaCl, 10mM Tris, 1mM EDTA, 1mM EGTA, 0.5% NP-40, 100mM sodium fluoride, 100mM sodium orthovanadate, 1 tablet of complete-mini EDTA-free protease inhibitor (Roche)). A small volume of the extraction buffer was added to tissue biopsies, homogenized, and left on ice for 30 minutes to allow complete digestion of tissue. The mixture was then centrifuged at 20,000 RPM for 15 minutes and the supernatant was transferred to fresh

tubes. Human melanoma cell lines and primary cell lysates were extracted using a RIPA buffer (50mM Tris-HCl ph8.0, 150mM NaCl, 1%NP-40 (Igepal), 0.5% sodium deoxycholate, 0.1% SDS, 100mM sodium fluoride, 100mM sodium orthovanadate, 1 tablet of complete-mini EDTA-free protease inhibitor (Roche)). RIPA buffer was applied to a monolayer of cells before lysing with a cell scraper. Solution containing cell lysate was then transferred to a microtube and left on ice for 15 minutes to allow complete digestion. The mixture was centrifuged at 12,000 RCF for 10 minutes at 4°C, and supernatant was transferred to a new microtube for protein quantification.

Cell line and primary cell protein lysates were quantified using the Pierce BCA assay and protocol (ThermoFisher). Protein lysates (50µg) were separated on a 10% SDS-PAGE gel (H_20, 10% Acrylamide, 370 mM Tris pH 8.8 bottom, 0.01% SDS, 0.01% APS, 0.004% TEMED) in a 1X SDS-PAGE running buffer at 120V. The gel was transferred onto a nitrocellulose membrane using the iBlot apparatus (Invitrogen). Membranes were placed in a blocking solution containing 3% BSA in 0.05% Tween-20 in 1XPBS for 1+ hours at room temperature, and then incubated with an affinity-purified custom-made rabbit polyclonal anti-human PANX1 antibody (1:1000; PANX1 CT-412) overnight at 4°C [74]. The membrane was additionally labeled with a mouse anti-human GAPDH antibody as GAPDH was used as a loading control protein. Membranes were labeled with two secondary antibodies, goat anti-rabbit AlexaFluor-680 and goat anti-mouse AlexaFluor-800 (Life Technologies). Membranes were washed three times with 0.05% Tween-20 in 1XPBS between antibody applications and imaged using an Odyssey infrared imaging system (Licor).

A peptide competitor was applied at a ratio of 1:50 (antibody:peptide; calculated from antibody and peptide concentrations) using a rabbit polyclonal anti-human PANX1 antibody (PANX1CT-412; 0.35µg/µL) and the corresponding human PANX1-peptide (6µg/µL)[74]. The peptide was combined with the antibody at room temperature for 30 minutes prior to membrane application. A separate tube containing the antibody without added peptide was also put at room temperature so methodologies of resulting Western blots were consistent. Representative samples for quantification in Figure 6A were blindly chosen to control for selection bias. One distant metastasis sample in Figure 6A

was removed for this analysis (resulting in N=3) due to protein degradation indicated by control GAPDH protein. A375 lysate was used to normalize protein expression between multiple Western blots.

Immunohistochemistry

Patient-derived melanoma sections of 14 primary tumours, 14 nodal tumours, and 6 distant metastasized tumours were provided by OICR. Pre-imaged tumours stained using H&E were additionally provided by OICR for each tumour. Paraffin embedded sections were deparaffinized using xylene and sequential dilutions of ethanol (100%, 95%, 70%, 50%, double distilled H_2O). Antigen retrieval was performed by submerging de-pariffinized sections in 1.5% of Vector Labs Antigen Unmasking Solution (Vector Laboratories) and heating for 5 minutes in a microwave at 80% power. Sections were labeled for PANX1 using a rabbit anti-human PANX1 polyclonal antibody (1:50; PANX1 CT-412) and a peptide-competition assay (1:50 molar excess calculated from antibody and peptide concentrations) was applied on selected samples to confirm the specificity of our antibody [74]. A rabbit anti-MITF antibody (1:100; Abcam) was used as a marker of melanoma cells. Goat anti-rabbit AlexaFluor488 was applied as a secondary antibody (1:400) and Hoechst33342 (1:1000; 16.2mM solution from Life Technologies) was used to label cell nuclei. PBS was used to wash sections between antibody applications. Slides were mounted using Vectashield mounting medium (Vector Laboratories) and nail polish, and refrigerated at 4°C for one day before imaging.

Immunocytochemistry

Cell lines and primary melanoma cells were grown to 70-80% confluency on glass coverslips. Cells were fixed by incubating with 80% methanol + 20% acetone for 15 minutes in 4°C. Cells were then washed twice with 1X PBS and incubated with a blocking solution (2% BSA, 1X PBS) for approximately one hour. The same antibodies described above were applied (PANX1 1:250, MITF 1:250). Coverslips were mounted using room temperature Airvol (Mowiol 4-88; Sigma Aldrich) and refrigerated at 4°C for one day prior to imaging.

Localization of PANX1 to the cell membrane in A375 and A375-MA2 cells was quantified from multiple immunofluorescence images depicting PANX1 expression in both cell lines. Percentage of cell membrane PANX1 expression was derived from the number of cells with the appearance of PANX1 at the cell surface divided by the total number of cells in each field. Cell identity during these analyses was not disclosed and results were averaged between individuals.

Microscopy

For migration assays, a Zeiss Axiovert A1 brightfield microscope (Carl Zeiss Inc.) was used for imaging A375 and A375-MA2 cell monolayers on gridded 35mm Petri dishes. For chick-CAM assays, $DiOC_{18}(3)$ (3,3'-Dioctadecyloxacarbocyanin Perchlorate) labeled A375-MA2 tumours were imaged using a Zeiss Lumar.V12 stereoscope with GFP filter and a 0.8X NeoLumar objective. All immunofluorescence labeled cells and tumour sections were imaged using a Zeiss LSM 800 Laser Scanning Fluorescence Confocal Microscope. The laser lines used include 405nm (DAPI), 488nm (Alexa488), and 561nm (Alexa555). The tiling module of Zen2.3 was used to image and stitch together large tumour sections.

WST-1 Cytotoxicity Assay

Cell Proliferation Reagent WST-1 (Sigma) was used to assess cytotoxic effect of CBX and PBN on A375 and A375-MA2 cells. In a 96-well plate, 500 A375 cells and 1500 A375-MA2 cells were seeded in a final volume of 100µL/well of serum media combined with increasing concentrations of CBX (10µM-1000µM), PBN (100µM-8.93mM), or 25µL of vehicle control and incubated at 37°C at 5.0% CO_2. On day five, 10µL of Cell Proliferation Reagent WST-1 was added to each well and further incubated at 37°C and 5.0% CO_2 for 30-45 minutes. The plate was then agitated on a vortex at low speed for one minute before measurements were taken on an Epoch microplate spectrometer (Biotek). UV absorbance of the sample was measured at 450nm and a reference wavelength of 690nm was subtracted from this measurement to account for signal background. Three biological replicates were obtained with each cell line.

Growth Curves

In a 6-well culture dish, 1.5 x 10^4 A375 and A375-MA2 human melanoma cells were seeded in each well at day 0 of the experiment. Serum-containing media with 1mM PBN, 100μM CBX, or HBSS control was added to individual wells. For 4 days starting at day 2, cells were separated from plate and re-suspended using 500μL of 0.25% trypsin-EDTA (Invitrogen). Cells (10μL) were transferred and mixed with 10μL of Trypan Blue stain solution (MP Biomedicals). From this mixture, 10μL was transferred to a reusable, glass cell counting chamber slide and cells counts were performed using an automated cell counter (Countess®) [38]. Two live cell counts of a single well were averaged each day. Three biological replicates were performed on each cell line.

Migration Assays

A375 cells were grown to confluency on gridded 35mm Petri dishes. Half of the monolayer of cells was removed using a cell scraper under sterile conditions and remaining cells were gently washed twice with 1mL of serum-free media to remove remaining debris. Serum-free media with 50μM of CBX, 1mM of PBN or HBSS control was applied to A375 cells. This protocol was modified slightly to include 10% FBS serum media and 100μM of CBX for A375-MA2 cells, since they did not tolerate the serum-free environment. Three pictures of individual squares on the gridded plate were taken immediately after initial scratch and 72 hours later using a Zeiss Axiovert A1 brightfield microscope. The x-distance travelled was determined by dividing the total area the cells migrated by length of the square grid on the plate (2mm) using ImageJ software [38]. Three biological replicates were performed.

Melanin Extraction

In a 60mm culture dish, 4 x 10^5 A375 and A375-MA2 human melanoma cells were seeded in serum media containing 100μM CBX, 1mM PBN or HBSS control. Three days later, cells were trypsinized and re-suspended in fresh serum media. One million cells were counted using an automatic cell counter (Countess®) and transferred from each treatment plate into separate microtubes and centrifuged for 10 minutes at 200 x g. After

media was aspirated from each tube, pellets were suspended in 200µL of 1M NaOH and 10% DMSO and incubated for 2 hours at 65°C. Samples were then mixed and immediately transferred in a 96-well plate and UV absorbance was read at 450 nm using an Epoch microplate spectrometer (Biotek) [38]. Three biological replicates were applied to each cell line.

Melanin standard curves were developed to determine the concentration of melanin extracted from one million melanoma cells based on UV absorbance values. Melanin (Sigma) dissolved in 1M NaOH and 10% DMSO was generously provided by Dr. Lina Dagnino (University of Western Ontario). Sample melanin concentrations (0µg/µL-100µg/µL) were developed using serial dilutions of stock melanin (20mg/mL) and transferred to a 96-well plate (200µL). UV absorbance was read at 450 nm using an Epoch microplate spectrometer (Biotek). Absorbance values were graphed against corresponding melanin concentrations on Microsoft Excel to develop the melanin standard curve. A line-of-best-fit was applied and an equation for this (developed using Microsoft Excel) was used to convert UV absorbance values into melanin concentrations (µg/µL) from each experiment.

Tumour Growth in the Chick Chorioallantoic Membrane Assay (chick-CAM assay)

Fertilized chicken eggs (McKinley Hatchery, St. Mary's, ON) were incubated in a rotating incubator at 37°C with 70% humidity for 3 days. Eggs were cracked and embryos were removed from the shell, transferred into weigh boats and covered with plastic lids. Embryos were incubated for 7 more days to allow for further growth and development. At day 10, a $DiOC_{18}$(3)(3,3'-Dioctadecyloxacarbocyanin Perchlorate) Lipophilic Cell Tracer (1:500) was applied to confluent A375-MA2 cells for 2 hours and then washed with 1X PBS. After, 20µL of serum-free media containing a PANX1 channel blocker or vehicle control and one million A375-MA2 cells were combined with 20µL of Matrigel (1:1) and implanted on bifurcating blood vessels in the CAM of the chicken embryo. Embryos were returned to incubators and tumours were treated directly with 40µL of treatment (PANX1 blocker or vehicle control) for 6 days. At day 17, pictures of tumours were taken using a stereomicroscope and tumours were excised and

weighed [38, 75]. Surface area analyses of the tumours were performed on ImageJ software using the 'freehand trace' tool.

Statistical Measures

All statistical analyses were performed using GraphPad Prism software (San Diego, CA). Student's t-test, one-way ANOVA followed by a Tukey's *post hoc* test, or two-way ANOVA with multiple comparisons followed by a Sidak test were conducted. Data error bars indicate mean ± s.e.m.

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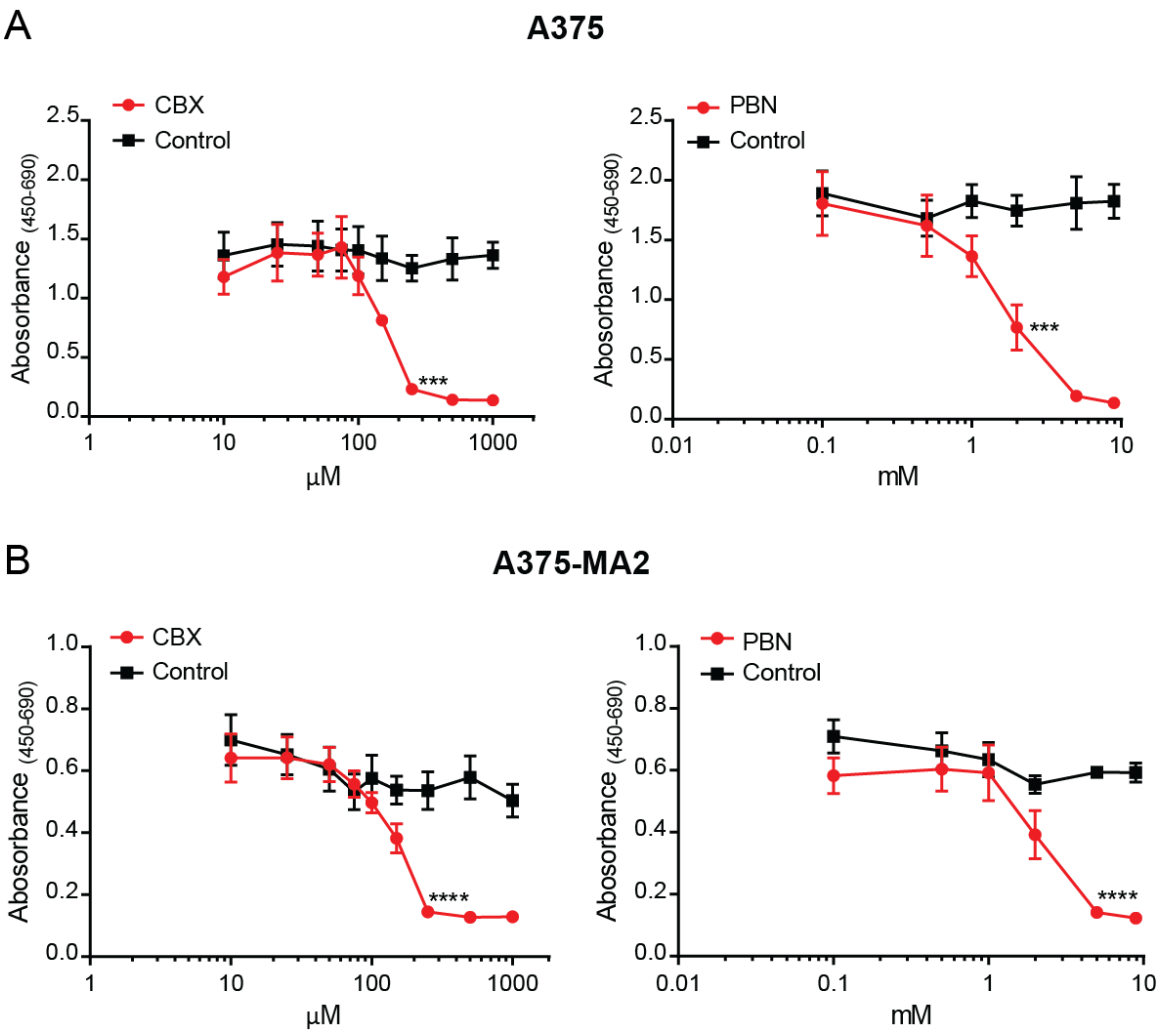
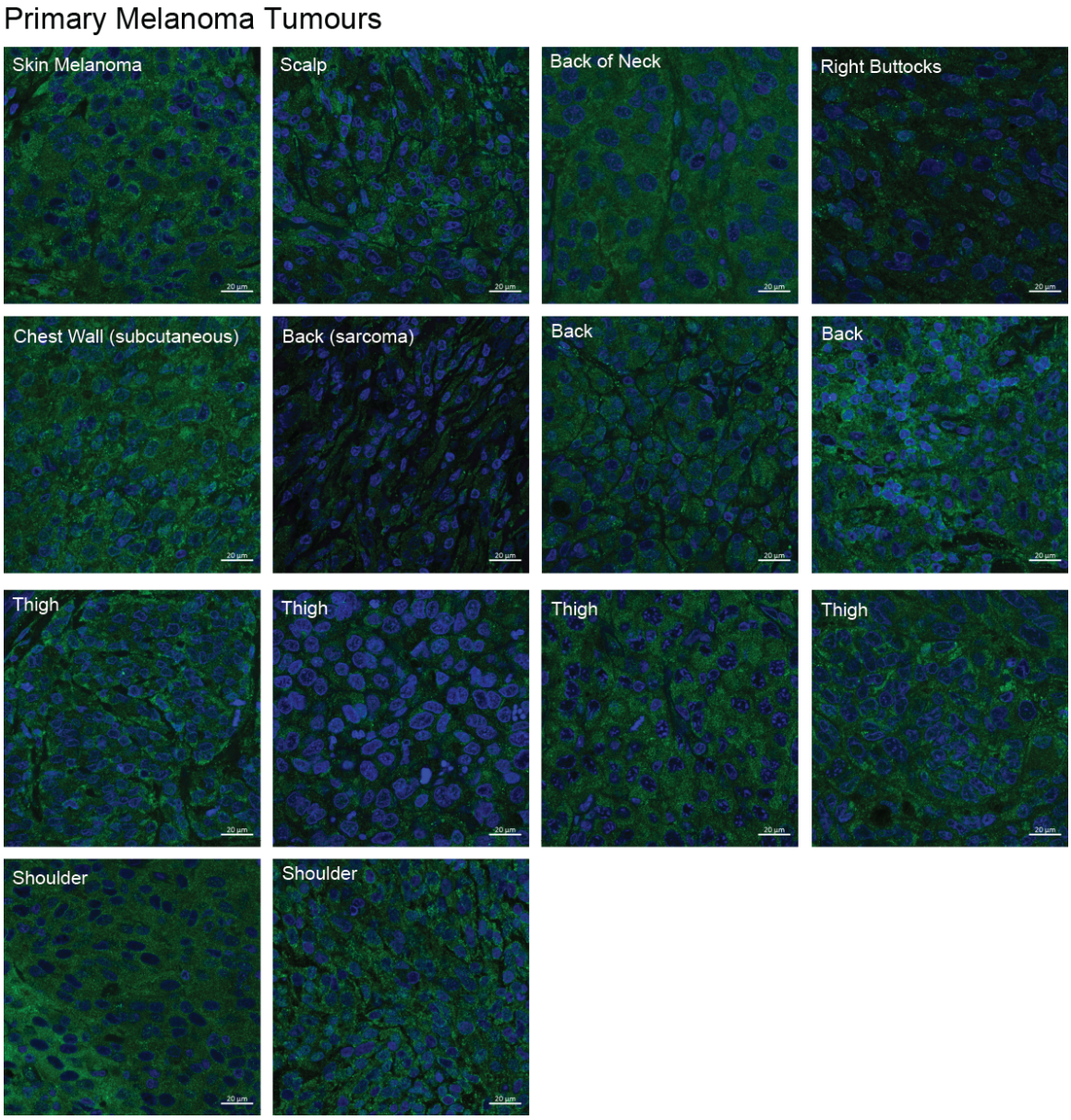
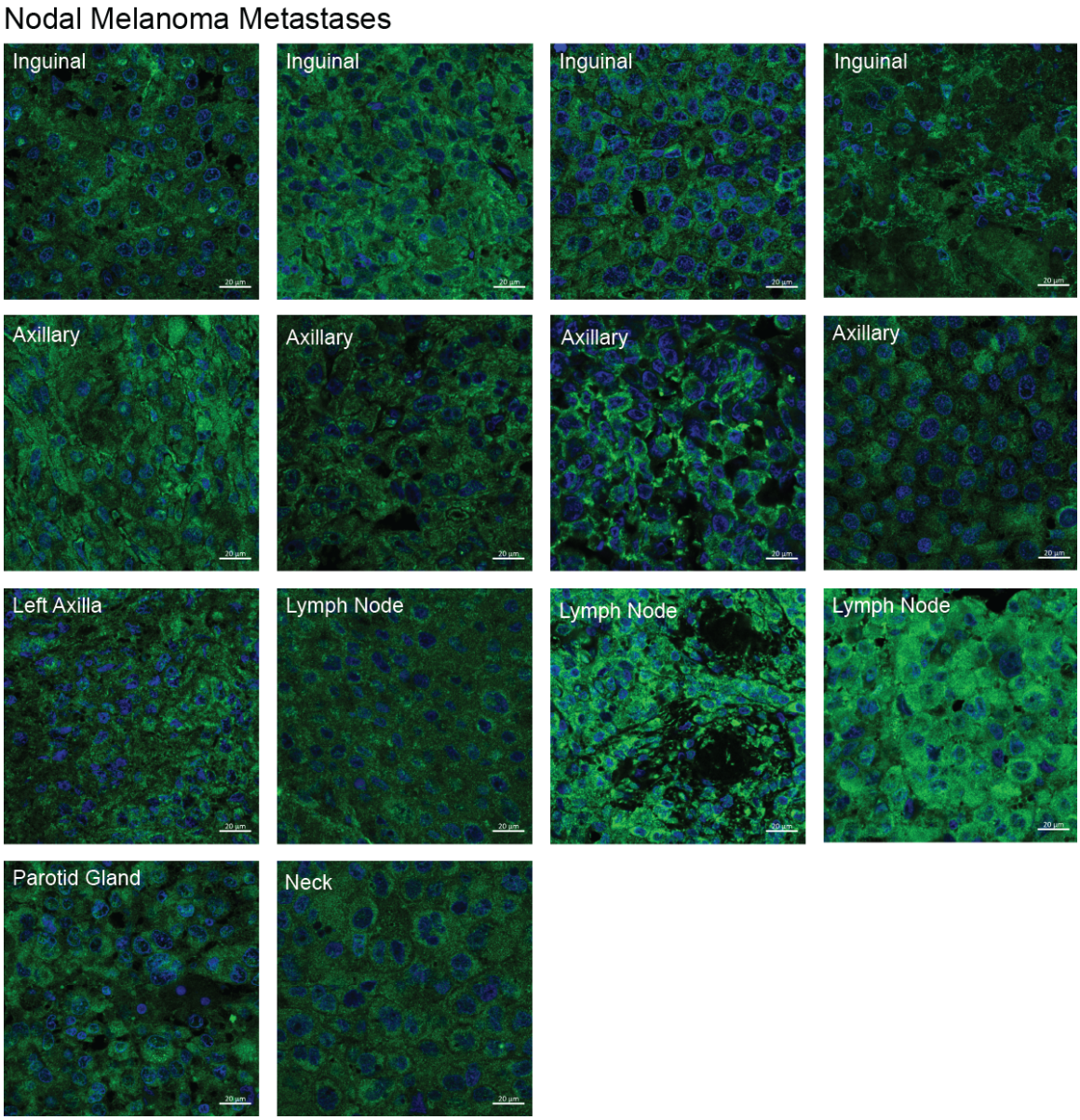
2.7 Supplemental Figures



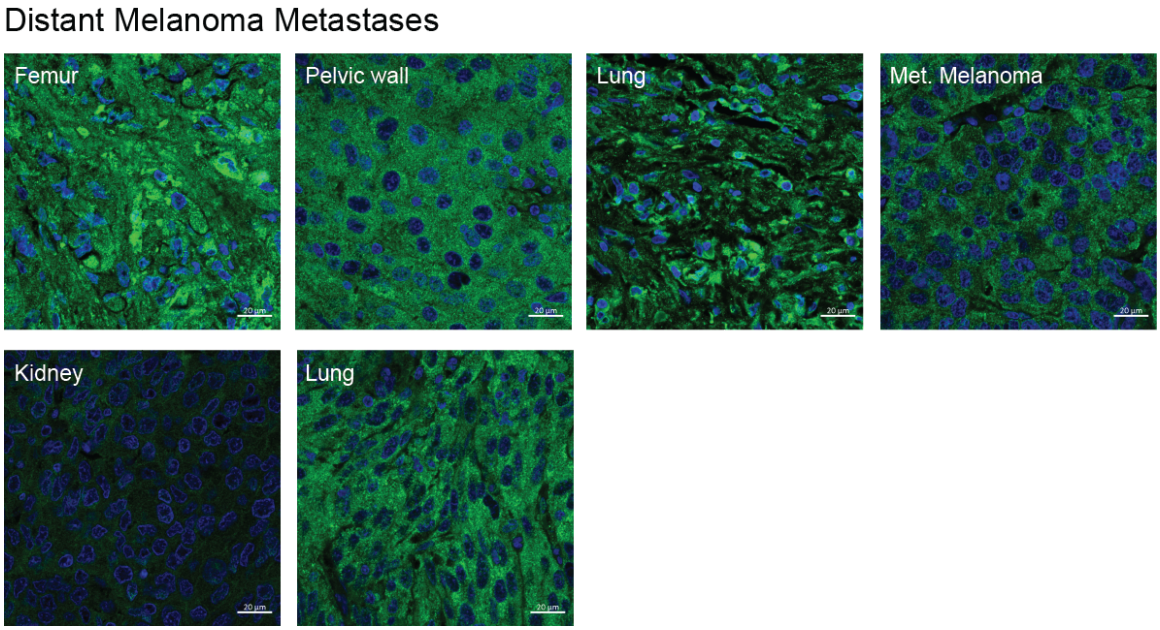
Supplemental Figure 1. A WST-1 cytotoxicity assay was used to assess A375 and A375-MA2 cell viability when CBX or PBN is applied. Cytotoxic effects do not occur at 100µM CBX and 1mM PBN indicating that results from *in vitro* and *in vivo* experimental assays are due to channel blockade rather than a decrease in cell viability. *A.* Significant cytotoxic effects are noted at (i) 250µM CBX or (ii) 2mM PBN in A375 melanoma cells. *B.* A significant decrease in cell viability occurs at (i) 250µM CBX or (ii) 5mM PBN in A375-MA2 melanoma cells. Statistical analyses were performed using a two-way ANOVA with multiple comparisons followed by a Sidak test, ***P<0.001, ****P<0.0001; Bars indicate s.e.m.



Supplemental Figure 2. Immunofluorescence of PANX1 expression in all patient-derived primary melanoma sections provided by OICR. Each panel represents a different patient. PANX1:green, Hoechst: blue; Scale: 20µm.



Supplemental Figure 3. Immunofluorescence of PANX1 expression in all patient-derived nodal melanoma metastases provided by OICR. Each panel represents a different patient. PANX1:green, Hoechst: blue; Scale: 20µm.



Supplemental Figure 4. Immunofluorescence of PANX1 expression in all patient-derived distant melanoma metastases provided by OICR. Each panel represents a different patient. PANX1:green, Hoechst: blue; Scale: 20µm.

Table 1. Melanoma tumour location of patient-derived melanoma biopsies used in Figure 6A, according to the OICR database.

Sample	**Location**	**Sample**	**Location**	**Sample**	**Location**
Primary 1	Scalp	**Nodal 1**	Left axillary node	**Distant 1**	Metastatic melanoma
Primary 2	Neck	**Nodal 2**	Axillary nodal	**Distant 2**	Right kidney
Primary 3	Shoulder	**Nodal 3**	Neck	**Distant 3**	Metastatic melanoma
Primary 4	Subcutaneous chest wall	**Nodal 4**	Neck		

Chapter 3

3 Discussion and Conclusions

3.1 Overall Study Conclusions

Changes in PANX1 expression, protein levels or channel function have been correlated to the onset or progression of most cancers, including melanoma [1]. The objective of this thesis was to evaluate PANX1 levels in human melanoma and determine its role in tumorigenic properties. Here, I have identified endogenous PANX1 levels in five human melanoma cell lines (A375-MA2, A375, A2058, C8161, 131/4-5B1) with PANX1 localized intracellularly and to the cell membrane. Preliminary work evaluating differences in PANX1 localization between A375 and A375-MA2 cells found a lower percentage of A375-MA2 cells labeling PANX1 at the cell membrane. PANX1 channels have been primarily characterized as ATP release channels at the cell surface, whereas channels localized to the ER can have a reported function in calcium release [2, 3]. Therefore, these results suggest that there may be potential differences in the localization and resulting function of PANX1 channels between the two cell lines; however, additional experiments including co-localization assays and cell surface biotinylation assays should be performed to confirm localization differences. I have also noted variable PANX1 protein levels in patient-derived melanoma biopsies from each stage of melanoma development, with PANX1 localized primarily to melanoma tumour cores and some stromal regions of the tumour. Furthermore, in collaboration with local LHSC surgical oncologist (Dr. Aaron Grant at University Hospital and Dr. Steve Latosinsky at Victoria Hospital, London, ON, Canada) I have identified comparable PANX1 levels and localization patterns in patient-derived primary melanoma cells to human melanoma cell lines using Western blot and immunofluorescence analyses.

To assess the role of PANX1 in the tumorigenic properties of human melanoma, I applied two Health Canada approved compounds that are commonly utilized to block PANX1 channels on A375 and A375-MA2 cell lines. By treating melanoma cells with either Carbenoxolone (CBX) or Probenecid (PBN), I found a significant reduction in cell

growth and migration. I have also discovered a significant increase in melanin production, which has been shown in previous studies to relate to a more melanocytic-like phenotype. The *in vivo* experiments using a chicken embryo model have additionally revealed a role for PANX1 in A375-MA2 tumour growth, as tumours treated with PANX1 channel blockers weighed significantly less than control tumours.

Overall, this data suggests that high PANX1 levels are associated with the malignant behaviour of human melanomas, which is consistent to the majority of cancer reports [1]. It is clear throughout the literature that the role of Panx1 as a tumour-suppressor or -promoter in cancer may vary depending on the species and cell-type examined. In particular, Panx1 was found to have opposing functions in cell growth and tumour formation between human glioma cells and rat C6 glioma cells [4, 5]. However, my current findings support previous research in BL6 mouse melanoma cells where Panx1 knockdown caused a reversion to a melanocytic-like phenotype [6]. Similar to my results, a reduction in Panx1 levels led to decreased cell migration and tumour growth on a chicken embryo model and a significant increase in melanin production. I have also provided support for CBX and PBN as available and effective treatment options to reduce the tumorigenic properties of melanoma. This supports previous research showing the therapeutic utility of PANX1 inhibition, where CBX treatment in mice was found to reduce the development of new distant breast cancer metastases [7]. The authors also found that ATP released from PANX1 channels could act on P2Y-purinergic receptors to suppress apoptosis in metastatic cells as they migrate through the vasculature. Although the mechanism is currently unknown, it may be possible that PANX1-related changes in cell growth, migration, melanin production and tumour formation in human melanoma are mediated through purinergic signaling and therefore requires further investigation.

Collectively, this work has identified PANX1 in all stages of human melanoma. Support is also provided for CBX and PBN as alternative treatment options that may have an additive effect by reducing melanoma tumour growth and migration when used in combination with current therapeutic strategies including immunotherapies, targeted therapies, or radiation.

3.2 Limitations and Future Directions

3.2.1 Limitations of the Study

This study is the first to identify endogenous PANX1 levels in human melanoma that is associated with cellular and tumorigenic properties of the cells. However, there are a few limitations that should be addressed from the study outlined in this thesis.

PANX1 appears to be dysregulated in human melanomas and may contribute to the malignant behaviour of the cells. However, it is unknown if there are changes in PANX1 levels during human melanocyte transformation or if mutations in *PANX1* modulate channel function. Previously, research has found low Panx1 levels in L10 mouse melanocytes and increased Panx1 levels across isogenic mouse melanoma cell lines [6]. During this study, I experienced difficulty obtaining non-transformed, primary human melanocytes and culturing them on plastic culture dishes due to their slow growth *in vitro*. Future investigations should evaluate levels of PANX1 in primary human melanocytes and compare these to levels described in our human melanoma samples to determine if a trend similar to what was observed in murine cells will occur. In addition, the Cancer Genome Atlas has reported six somatic mutations (Q264* and T176I: 121 samples analyzed [8]; H190Y, S239L, G168E, and Y150F: 479 samples analyzed [9, 10]) on the *PANX1* gene from a subset of melanoma tumours. It is therefore possible that these mutations may lead to changes in channel localization or channel function that enhance the malignant properties of melanoma in a similar manner to mutations found in metastatic breast cancer cells [7]. Future studies should evaluate the presence of each mutation in human melanoma cell lines and patient samples, and characterize each mutation to determine if they lead to changes in cell growth, migration or melanin production.

Previous reports have shown that Panx2 and Panx3 may compensate for reduced levels of Panx1. Both Panx1 and Panx2 need to be ablated for neuroprotection to occur in a model for ischemic stroke since both pannexins can compensate for one another [11]. In addition, Panx3 is up-regulated in the skin of Panx1-null mice [12]. Although I have

shown endogenous PANX1 levels in all human melanoma cell lines and biopsies, I have not examined the presence of PANX2 and PANX3 levels before and after PANX1 channel blockade. Previously, members from our group did not detect the presence of Panx2 and Panx3 in mouse melanoma cells [6]. Since trends may differ between species, future work should evaluate if other pannexin species are present in human melanoma cell lines and patient samples. If present, additional studies could be performed to assess any possible compensation by other pannexins when PANX1 channel blockers are used on human melanomas.

By applying CBX and PBN to block PANX1 channels, I could detect reduced cell growth, migration and reduced tumour growth in two human melanoma cell lines. PBN has been reported to preferentially block PANX1 channels, although recent investigations have indicated that P2X7 receptors may also be blocked using this compound [13]. In comparison, CBX inhibition of PANX1 channels is dose-dependent [14, 15]. Low concentrations of CBX (1-10μM) significantly reduce electric currents through Panx1 channels [15]. However, at concentrations equal to or greater than 100μM, the effect of CBX is less specific and has been found to weakly inhibit connexin 46 (Cx46) channels in addition to Panx1 channels [15]. Future experiments should assess the presence of Cx46 and other connexin hemichannels in human melanoma cells to determine if changes to cell growth, migration and melanin production are solely due to PANX1 channel blockade. Alternatively, investigations could include the ^{10}PANX1-peptide [7, 16] and anti-PANX1 antibodies [17] as more specific approaches to target PANX1 channels thereby limiting potential off target effects of each compound.

To evaluate changes in migration of the A375-MA2 cells, the protocol for the scratch assays was modified to include full-serum media (10% FBS) due to their intolerance to serum-free conditions. With the addition of serum, it is possible that differences I detect from this assay are not solely due to cell movement, but rather a combination of cell movement and proliferation. Since the A375-MA2 cells are sensitive to this experiment, cell attachment and motility could be enhanced by coating plastic culture dishes with components of the extracellular matrix including laminin-332 [18]. It may also be beneficial to utilize alternative methods including the transwell migration assays to assess

cell movement and invasion or a timelapse videomicroscopy to assess haptotactic motility of the cells.

In addition, preliminary results indicate less PANX1 at the cell surface of A375-MA2 cells compared to A375 melanoma cells. It will be important for future experiments to confirm these findings using cell-surface biotinylation assays as PANX1 localization may provide insight into the potential function of the channel (e.g., ATP release at cell surface or intracellular ER calcium channel [2, 3]).

Finally, future studies should focus on identifying the cellular mechanism that involves the role of PANX1 in cell growth, cell migration, melanin production and tumour formation in malignant melanoma. Future work could assess the involvement of purinergic signaling in the tumorigenic properties of human melanoma cells by using ATP release assays to determine if this nucleotide is released by PANX1 channels [19]. P2X-purinergic inhibitors including suramin could also be applied to melanoma cells to see if there is a similar reduction in cell growth, migration and tumour size [20]. In addition, previous work identified a reduction in β-catenin levels when Panx1 shRNA knockdown was applied to the BL6 mouse melanoma cell line [6]. Therefore, changes to molecules involved directly in or downstream of the Wnt/ β-catenin signaling pathway should also be evaluated through future experiments to determine if PANX1 is modulating cell growth and melanin production through this pathway.

3.2.2 Preliminary Results: Evaluation of A375-MA2 metastasis to distant organs using the chick-CAM assay

In Chapter 2, the chick-CAM (chicken chorioallantoic membrane) assay was used to evaluate changes to A375-MA2 tumour growth when PANX1 channel blockers are applied. I further utilized this model to evaluate the ability of A375-MA2 cells to metastasize to distant organs of the chicken embryo including the lungs, liver, and brain. A control organ from an uninoculated embryo and an organ from an A375-MA2-inoculated embryo were initially examined to evaluate the extent of metastasis. There was no significant difference in *Alu* expression between the two experimental groups,

suggesting that the cells did not metastasize to the lungs, liver or brain of the chicken embryo (Appendix B). To confirm the lack of melanoma cell metastases, raw Ct_{Alu} values from qPCR experiments were compared to the standard curves developed for each organ (Appendix C). Ct_{Alu} values from qPCR experiments were comparable to those representing approximately zero cell metastases on the standard curve of each corresponding organ. This indicates that *Alu* amplification found in experimental uninoculated and inoculated chicken embryos are merely background *Alu* expression.

The chick-CAM assay is commonly used in the literature to evaluate cancer cell metastasis [6, 21]. However, most reports inoculate cells directly onto the chick-CAM under a coverslip. In this study, I combined melanoma cells with Matrigel so PANX1 channel blockers could be applied directly to the tumours. I suspect that the addition of Matrigel may delay the process of A375-MA2 cell intravasation and metastasis to distant organs of the chicken embryo. These processes already occur under a short period of time as A375-MA2 cells only have 6-7 days to grow following the initial inoculation. Therefore with the added delay, this may not be enough time for cells to produce collagenase, break through the Matrigel, and travel to distant chick organs. Most reports that utilize Matrigel on this experimental model evaluate cell differentiation or changes in angiogenesis [22]. Future work should apply A375-MA2 cells directly onto the chorioallantoic membrane of the chicken embryo to determine if this cell line is able to metastasize without the possible effects of Matrigel. A silicone ring [23] could also be utilized in future experiments to allow the direct inoculation and treatment of melanoma cells on the chick-CAM assay.

3.3 Summary

In summary, I have discovered endogenous PANX1 in multiple human melanoma cell lines, and at each stage of melanoma progression in patient-derived melanoma biopsies and primary cells, supporting PANX1 as a potential target for melanoma therapy. By applying CBX and PBN, two Health Canada approved compounds known to block PANX1 channels, multiple cellular and tumorigenic properties of human melanomas were reduced including cell growth, cell migration and tumour growth on a chicken

embryo model. There was also a significant increase in melanin production following PANX1 blockade, indicating a potential change to a more melanocytic-like phenotype. Taken together, these results indicate endogenous PANX1 levels in each stage of melanoma progression that is associated with the malignant behaviour of the cells. This work also supports PANX1 channel blockers as alternative therapeutic options that may be used in addition to current treatment strategies to increase the effectiveness of melanoma therapy.

3.4 References

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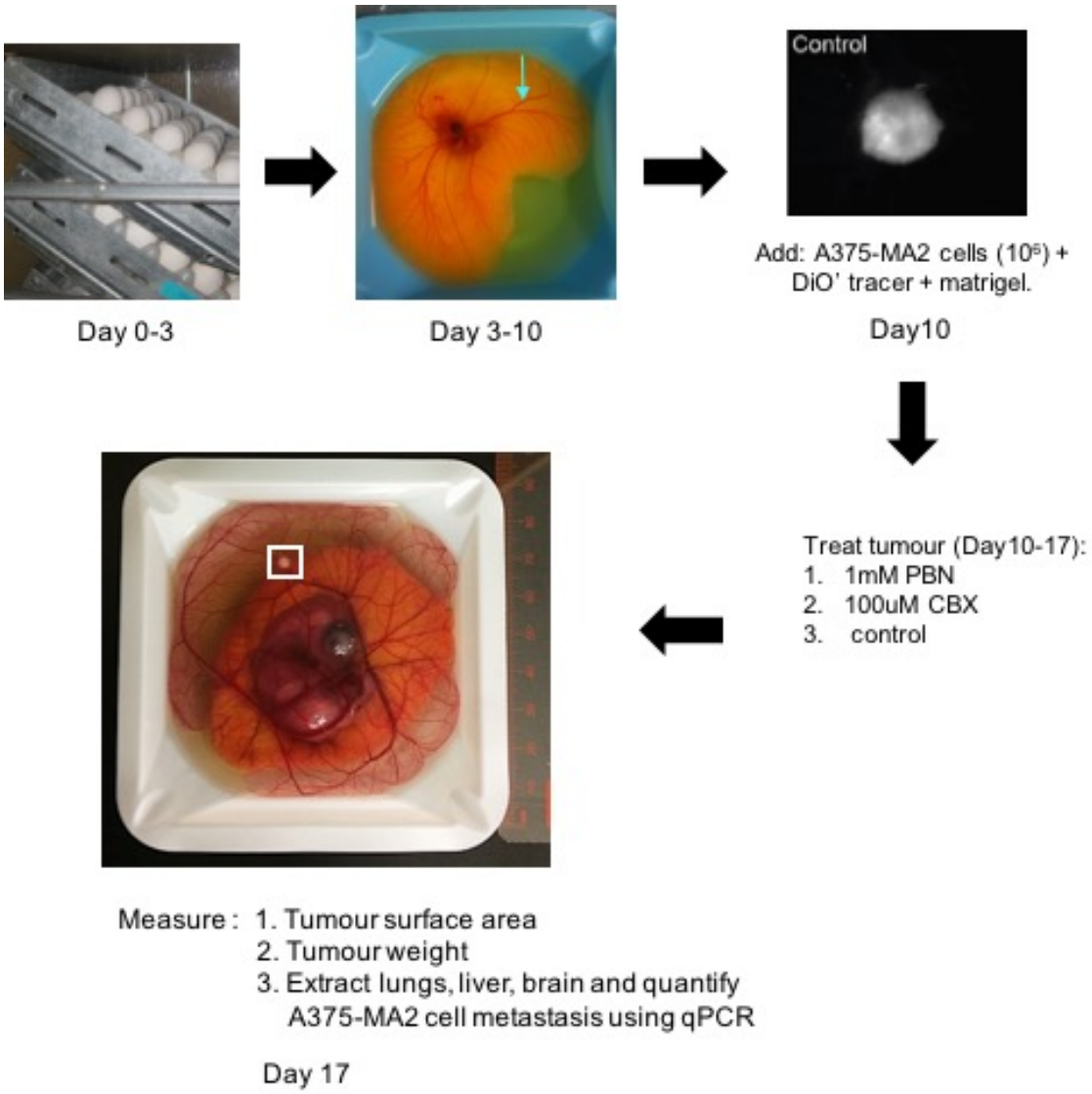
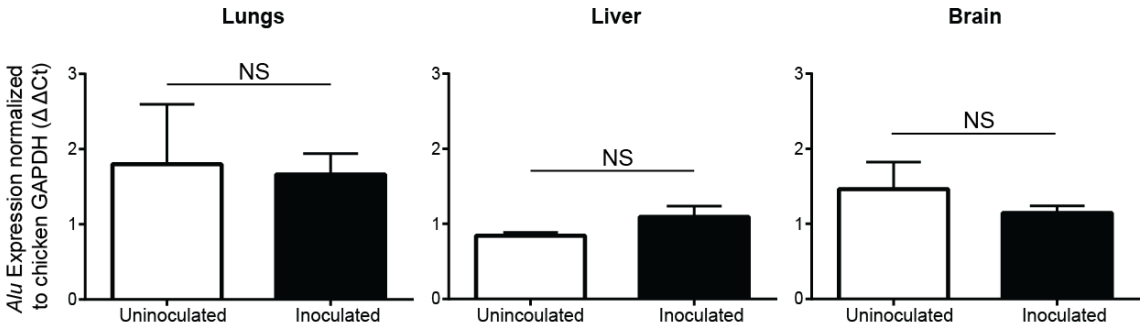
Appendices

Methods: Quantification of A375-MA2 melanoma cell metastases using the chicken chorioallantoic membrane (chick-CAM) assay.

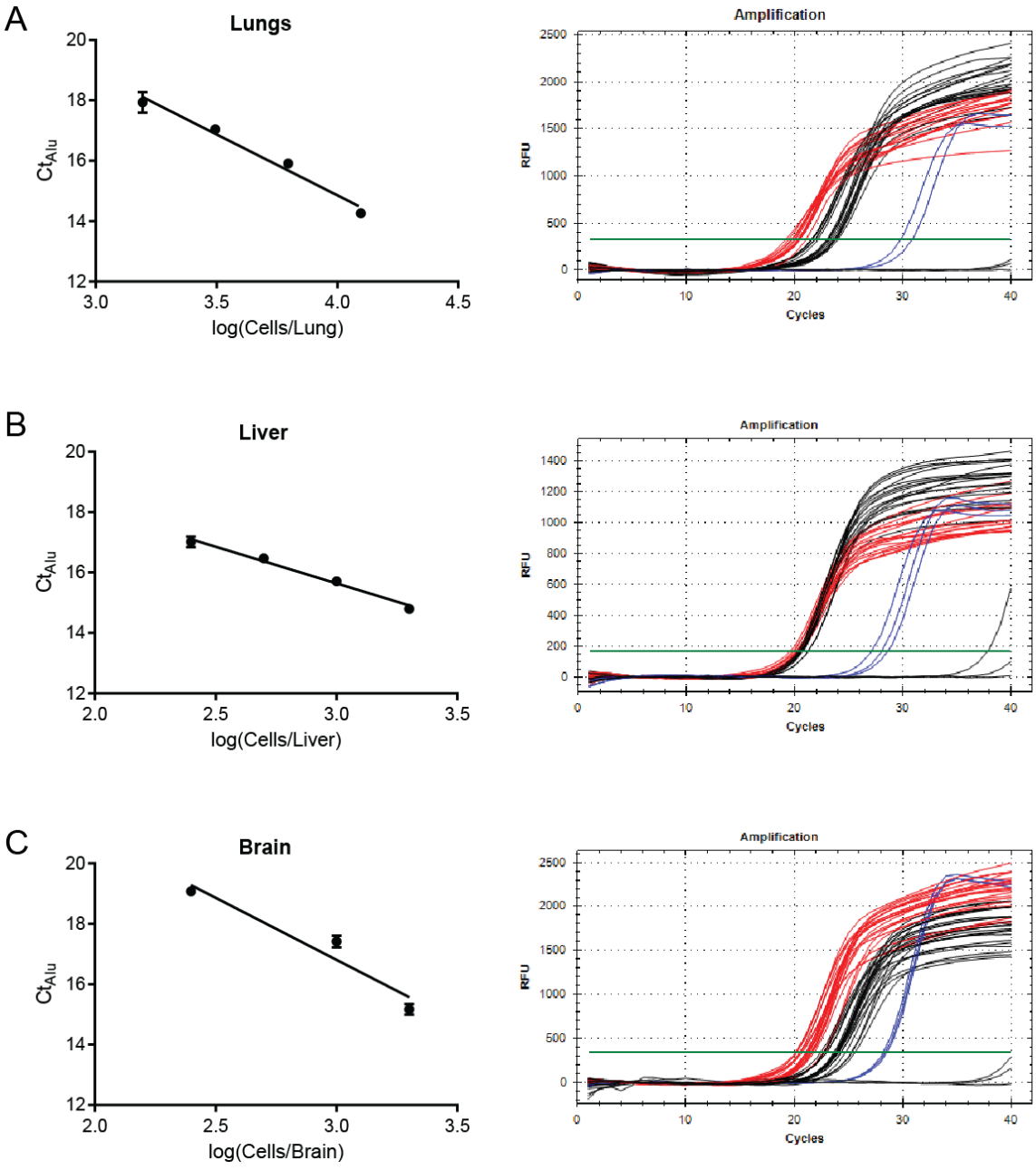
To perform this assay, the lungs (35µg), livers (50µg) and brains (50µg) of each embryo were harvested on the day 18 of the chick-CAM experiment and stored at -80°C. To quantify A375-MA2 cell metastasis, genomic DNA was extracted from each organ using the Sybr Green Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich) and a protocol provided by the Lewis Lab (Edmonton, AB). For qPCR, 10µM of human *Alu* primers (Forward: 5'-ACGCCTGTAATCCCAGCACTT-3'; Reverse: 5'-TCGCCCAGGCTGGAGTGCA-3') or chicken GAPDH primers (Forward: 5'-GAGGAAAGGTCGCCTGGTGGATCG-3'; Reverse: 5'-GGTGAGGACAAGCAGTGAGGAACG-3') were combined with 2x SYBR Green Supermix and 50X-diluted genomic DNA sample. Reactions were performed in a CFX96 LightCycler (BioRad) and graphs of amplification results were developed using the Bio-Rad CFX Manager 3.1 software. Standard curves were developed as positive controls and to quantify the numbers of melanoma metastases to each organ. These curves were developed from the genomic DNA extracted from serial dilutions of A375-MA2 human melanoma cells combined with lung (35µg), liver (50µg) or brain (50µg) tissue from uninoculated embryos. The log(number of cells/organ) was graphed against Ct_{Alu} values and linear regression was applied using GraphPad to develop a line of best fit. The equation to this line was developed using GraphPad and can be used to determine the exact number of cells in each organ using the Ct_{Alu} values from each qPCR experiment.



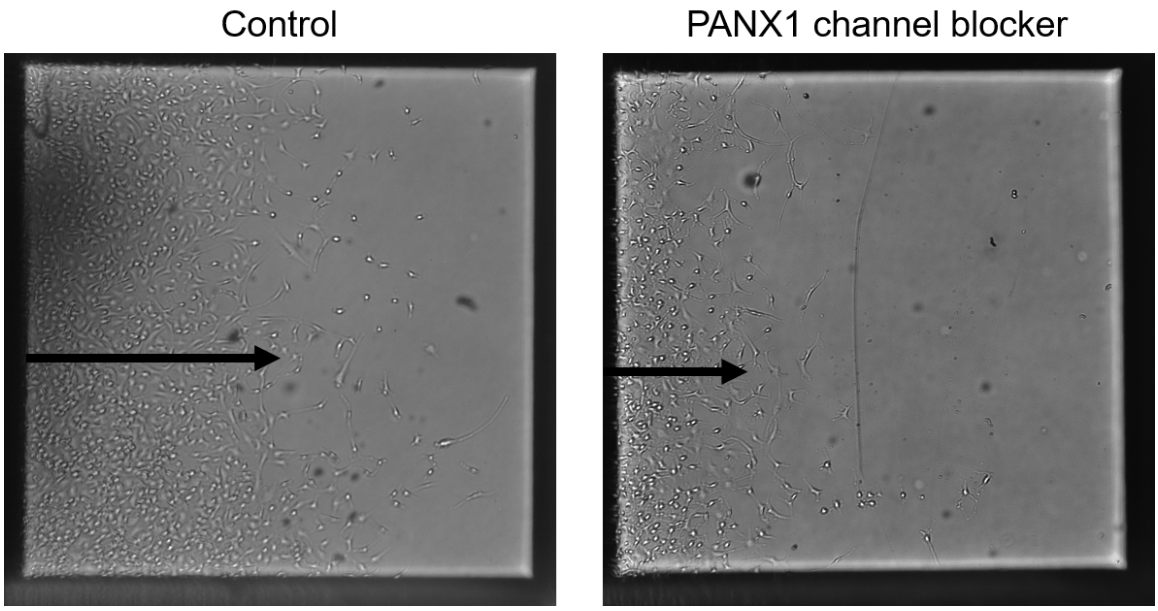
Appendix A. Chicken chorioallantoic membrane (CAM) assay. Day 0-3: Incubate fertilized eggs in a rotating incubator. Day 3-10: Crack embryos into a weigh-boat and allow embryos to develop in the incubator for one week. Day 10: Combine one million A375-MA2 cells labeled with a DiO' lipophilic tracer and Matrigel, and seed directly onto bifurcating blood vessels of the CAM. Day 10-17: Treat tumours with PANX1 channel blockers or vehicle control every day for one week. Day 17: Measure tumour surface area and weight, and extract the lungs, liver, brain of the chicken embryo and quantify for A375-MA2 cell metastasis using qPCR quantification.



Appendix B. A375-MA2 cells do not metastasize to the lungs, liver or brain of a chicken embryo model. Genomic DNA was extracted from the lungs, liver and brain of chicken embryos that were uninoculated or inoculated with one million A375-MA2 cells. *Human Alu* expression from qPCR experiments was normalized to *chicken GAPDH* expression in each sample. Final graphs were normalized to zero. There is no significant difference in *Alu* expression of organs extracted from inoculated or uninoculated chicken embryos. Lungs: Uninoculated N=2; Inoculated N=4, Liver: Uninoculated N=2; Inoculated N=5, Brain: Uninoculated N=3; Inoculated N=6. Statistical analysis includes student's t-test. Black bars: one million cells plus Matrigel, White bars: no cells added.


A
Lungs
Ct_{Alu}
log(Cells/Lung)
Amplification
RFU
Cycles
B
Liver
Ct_{Alu}
log(Cells/Liver)
Amplification
RFU
Cycles
C
Brain
Ct_{Alu}
log(Cells/Brain)
Amplification
RFU
Cycles

Appendix C. Standard curves and experimental amplification data for A375-MA2 cells in the lungs, liver and brain of chicken embryo. *A.* To develop the standard curve for A375-MA2 lung metastases, serial dilutions of A375-MA2 cells in 35μg of lung tissue: 12500, 6250, 3125, 1563, 0. The log(number of cells/lung) was graphed against Ct_{Alu} values. Linear regression was applied using GraphPad to develop a line of best fit; R^2:0.981 , y = -4.036x + 31. Amplification curves from lung qPCR experiment. *B.* The standard curve for liver metastasis was developed using serial dilutions of A375-MA2 cells in 50μg of liver tissue: 2000, 1000, 500, 250, 0. The log(number of cells/liver) was graphed against Ct_{Alu} values. Linear regression was applied; R^2:0.9685 , y = -2.424x + 22.91. Amplification curves from liver qPCR experiment. *C.* The standard curve for brain metastasis was developed using serial dilutions of A375-MA2 cells in 50μg of brain tissue: 2000, 1000, 500, 250, 0. The log(number of cells/brain) was graphed against Ct_{Alu} values. Linear regression was applied; R^2:0.9201 , y = -4.107x + 29.13. Amplification curves from brain qPCR experiment. Red: *Alu* expression from genomic DNA of chick organ, Black: *chicken GAPDH* expression from genomic DNA of chick organ and no template control, Blue: *Alu* expression from no-template control (negative control).



Appendix D. Migration Assay. Images taken 3 days following the initial scratch and application of PANX1 channel blockers or vehicle control. Cells treated with PANX1 channel blockers migrate less than cells treated with the control.

Curriculum Vitae

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Post-secondary Education and Degrees:

Queen's University
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2011-2015 B.Sc.

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Honours and Awards:

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Western Graduate Research Scholarship
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The University of Western Ontario
2016

Centre for Translational Cancer Research (CTCR)- Travel Award
Schulich School of Medicine and Dentistry
The University of Western Ontario
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Invited Oral Presentations:

1. *Inhibition of PANX1 reduces the tumorigenic properties of human melanoma.* 11th Annual Nexin Research Forum. The University of Western Ontario, London, ON. May 26th, 2017.
2. *Inhibition of Pannexin 1 reduces the malignant properties of human melanomas.* Anatomy and Cell Biology Research Day. The University of Western Ontario. London, ON. October 21st, 2016.
3. *Pannexin 1 as a novel regulator of malignant melanoma.* 10th Annual Nexin Research Forum. The University of Western Ontario. London, ON. February 18th, 2016.

Poster Presentations:

1. **Freeman T,** Harland L, Johnston D, Nouri-Nejad D, Brooke O'Donnell and Penuela S (2017). Inhibition of PANX1 reduces the malignant properties of

human melanomas. Oncology Research and Education Day, London, ON. June 16th, 2017. Poster number 77.

2. **Freeman T,** Harland L, Johnston D, Nouri-Nejad D, Brooke O'Donnell and Penuela S (2017). Inhibition of PANX1 reduces the malignant properties of human melanomas. London Health Research Day, London, ON. March 28th, 2017. Poster number 38.
3. **Freeman T,** Harland L, Johnston D, Nouri-Nejad D, Brooke O'Donnell and Penuela S (2016). Inhibition of PANX1 reduces the malignant properties of human melanomas. Proceedings of the American Society of Cell Biology Annual Meeting. San Francisco, CA. December 3-7th, 2016. Abstract program number B509/P1940.
4. **Freeman T,** Harland L, Johnston D, Nouri-Nejad D, and Penuela S (2016). Inhibition of PANX1 reduces the malignant properties of human melanomas. Oncology Research and Education Day, London, ON. June 17th, 2016. Poster 16.
5. **Freeman T,** Harland L, Nouri-Nejad D, Johnston D, and Penuela S (2016). Inhibition of PANX1 channels reduces malignant properties of human melanomas. London Health Research Day, London, ON. March 29th, 2016. Poster number 7.
6. **Freeman T,** Harland L, Nouri-Nejad D, Johnston D, and Penuela S (2015). Inhibition of PANX1 channels reduces malignant properties of human melanoma. Anatomy and Cell Biology Research Day, London, ON. October 23rd, 2015.